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Identifying temperament in dairy cows

A longitudinal approach

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CHAPTER 1

General introduction

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Personality and temperament

The concept of personality has a long history in human psychology. Broadly defined, human personality refers to those qualities of the individual that describe and account for consistent patterns of feeling, thinking, and behaving (see Funder, 2004; Larsen and Buss, 2005; Pervin et al., 2005). Personality is believed to consist of multiple characteristics or traits, including uniquely human ones related to attitudes, beliefs and identity (such as “self-esteem”, “locus of control” or “self-consciousness”) as well as behavioural traits that humans share with animals (such as, for example, “fearfulness”, “activity” or “aggression”) (Buss, 1989; Zuckerman, 1991; Cloninger et al., 1993; Bouchard and Loehlin, 2001; Funder, 2001). In the human literature characteristics of the latter category are often labelled as temperamental traits or dimensions, on the assumption that temperament is a less inclusive concept than personality (Strelau, 1982; Goldsmith et al., 1987; Zuckerman, 1991; Cloninger et al., 1993). Temperament is widely thought to represent the inherited foundation for personality, and is generally characterized by the following features (see Buss and Plomin, 1984; Goldsmith et al., 1987; Zuckerman, 1991; Gabbay, 1992; Strelau, 1998; Rothbart et al., 2000): (i) it is manifested early in childhood, and is relatively stable throughout development into adulthood, (ii) it is primarily biologically based, i.e., it is determined by genetic and neurobiological mechanisms, (iii) it mainly refers to formal characteristics of behaviour and behavioural reactions such as intensity, amplitude, speed, energy, response threshold, latency and recovery time etc., and (iv) it is most clearly expressed in novel and unpredictable situations.

Described in this way, human temperament is closely related not only to the concept of animal temperament (see Lyons, 1992; Clarke and Boinski, 1995; Réale et al., 2007; Archard and Braithwaite, 2010) but also to other concepts of individual differences in animals such as fearfulness (Boissy, 1995; Jones, 1996), coping style (Mason, 1984; Koolhaas et al., 1999, 2007, 2010), and behavioural syndrome (Sih et al., 2008a, b). Because of these apparent similarities between human and nonhuman species, there is an increasing interest in comparative cross-species temperament and personality research (see Gosling and John, 1999; Gosling, 2001; Carere and Eens, 2005; Carere et al., 2010; Nettle and Penke, 2010). At the same time, however, there is a lack of consistency in the literature regarding the terminology used to categorize specific behavioural characteristics of the individual. Among animal researchers, for example, the terms “personality”, “temperament”, and “coping style” are frequently used

interchangeably (see Erhard and Schouten, 2001; Visser, 2002; Van Oers, 2003; Bolhuis, 2004; Mehta and Gosling, 2008; Gibbons, 2009; Melotti et al., 2011). Correspondingly, the precise distinction between an individual trait and an aggregate construct comprising multiple traits is not always clear. Coping style, for example, was considered an individual temperamental dimension by some authors (Steimer et al., 1997; Koolhaas et al., 2007, 2010), but a personality type, in which different traits are systematically linked, by others (Erhard and Schouten, 2001, Melotti et al., 2011).

Similarly, characteristics such as fearfulness or aggression were either thought to represent single temperamental traits (Gosling, 2001; Réale et al., 2007), or to refer to behavioural syndromes which involve “suites” of correlated traits (Sih et al., 2004a). Nevertheless, and regardless of terminology, consistency of individual behavioural differences across context and over time is universally and unequivocally accepted as key empirical evidence in support of the existence of temperament or temperamental traits.

Relevance of temperament for domestic animals

Studies in both humans and animals have provided a wealth of data showing that personality and temperament characteristics may affect a wide range of biological mechanisms and processes, as well as health and other life-outcomes related to, for example, the formation of social bonds and relationships, reproduction, survival, and longevity (see Koolhaas, 1994; McEwen, 2001; Cavigelli, 2005; Korte et al., 2005; Mehta and Gosling, 2008; Smith and Blumstein, 2008; Archard and Braithwaite, 2010; Carere et al., 2010; Nettle and Penke, 2010; Capitanio, 2011; Kern and Friedman, 2011). In the context of domestic animal production, this means that temperament may influence the ability of farm animals to cope with the environmental conditions they are subjected to, and, thereby, their health and welfare (Wiepkema, 1984, 1987; Schouten and Wiepkema, 1991; Boissy, 1995; Wechsler, 1995; Jones, 1996; Blokhuis et al., 1998; Broom, 2001; Erhard and Schouten, 2001). This recognition has profoundly stimulated research in the field of farm animal temperament. Results of this work have the potential to change husbandry and breeding practices in order to prevent or alleviate adverse consequences, and to improve farm animal welfare (see Burrow, 1987; Mendl and Deag, 1995; Faure and Mills, 1998; Jones and Hocking, 1999; Erhard and Schouten, 2001; Mormède et al., 2002; Bolhuis, 2004; D’Eath et al., 2010).

Temperament in dairy cows

The current thesis is concerned with temperament of dairy cows. It specifically builds on previous work where individual differences in behavioural and physiological responses of (dairy or beef) cattle were studied with the use of various behavioural tests intended to induce a (mild) stress response (Kilgour, 1975; Boissy and Bouissou, 1995; Hopster, 1998; Grignard et al., 2001; Watts et al., 2001; Müller and Schrader, 2005; Kilgour et al., 2006; Gibbons et al., 2009a). Frequently used tests in these studies included so-called “open field”, “novel object”, “response to human”, and “restraint” tests, all of which are basically adaptations of tests originally developed for laboratory rodents (Archer, 1973), and that have been applied to different farm animal species (Manteca and Deag, 1993). The open field test involves the separation of an individual animal from its pen or herd mates and its subsequent confinement in a novel arena (“open field”) for a brief period (usually 5-10 minutes). During a novel object or response to human test, an individual animal is confronted with an unfamiliar object or a person, either in the home environment or in a test arena (similar to an open field). A restraint test is meant to (more or less drastically) limit the movement of an animal for a brief period of time, for example by tethering, or by close confinement in a weighting crate or crush. These tests allow for the objective, accurate and detailed recording of behavioural patterns that individual animals exhibit in response to controlled environmental challenges. Hence, they provide ideal tools to study dairy cow temperament. Therefore, submitting animals to these behavioural tests was adopted as the principal experimental method in the present thesis.

Consistent differences in responsiveness of dairy cattle to behavioural tests were found in a number of studies (Boissy and Bouisou, 1995; Hopster, 1998; Müller and Schrader, 2005). This supported the idea that differential responses of individual animals reflected the existence of stable temperamental characteristics. However, observations were only made either in heifers (Boissy and Bouissou, 1995) or in adult cows across successive lactations (Hopster, 1998; Müller and Schrader, 2005). Thus, little is known about the long-term developmental stability of putative temperamental traits in dairy cows, from infancy into adulthood.

In several farm animal species, particularly poultry and pigs, links have been made between, on the one hand, temperamental traits and, on the other, measures related to important biological functions such as growth, immune resistance and reproduction (see Hessing et al., 1994; Van Erp – van der Kooij et al., 2000; Bolhuis et

al., 2003; Janczak et al., 2003a; Marin et al., 2003; Uitdehaag et al., 2008a). The search for similar relationships in dairy cattle has received limited attention so far. Hopster et al. (1998) reported an intriguing association in dairy cows between the physiological response to an open field test (in terms of the release of the adrenal stress hormone cortisol) and the immune response pattern to an endotoxin challenge. However, more data are needed to further substantiate the relevance of temperament to the dairy cow's capacity to adapt to its actual husbandry environment.

Although open field, novel object, response to human, and restraint tests have been extensively used in (dairy) cattle and other farm animal species, there is ongoing controversy about the interpretation of individual differences found in these (and other) behavioural tests (e.g., Piovezan et al., 1998; Rushen, 2000; Forkman et al., 2007). More specifically, the same behavioural measures are often interpreted quite differently by different authors. For example, vocalisations emitted by dairy cattle in an open field were thought to reflect fear in one study (De Passillé et al., 1995), but social calls in another (Müller and Schrader, 2005a). Consistent individual differences in open field vocalisations would then be attributed to the temperamental trait "fearfulness" according to the first interpretation, but to "underlying sociality" (i.e., the motivation to be near conspecifics, see Gibbons et al., 2010) according to the latter. Adding to the confusion are contradictory interpretations about the amplitude of response in relation to the strength of the emotion or motivation involved. Again, open field vocalisations of dairy cattle may serve as an example: both high (De Passillé et al., 1995) and low (Hopster, 1998) numbers of vocalisations were suggested to indicate high levels of fear. Rigorous experimental validation steps would be necessary to eliminate such inconsistencies. These include, among others: (i) establishing correlations between behavioural measures and physiological and neuroendocrine ones that are known to be activated in reaction to (specific) environmental challenges (see Schrader and Todt, 1998; Réale et al., 2007; Koolhaas et al., 2010), and (ii) using pharmacological tools to assess, for example, the sensitivity of a behavioural measure to treatment with anxiolytic (i.e., fear-reducing) drugs (e.g., Marin et al., 1997; Andersen et al., 2000a; Sandem et al., 2006). Until now, the physiological and pharmacological validation of behavioural tests in dairy cattle has only been addressed to a limited extent (see Forkman et al., 2007).

Aims of the thesis

The main aim of the present thesis was to examine the consistency of individual differences in behavioural and physiological responses to acute stressors in dairy cattle

in a longitudinal fashion, i.e. from early age until adulthood. Therefore, individual dairy cattle were repeatedly submitted to behavioural tests, i.e., during rearing as (young) calves, as pregnant heifers and during first lactation. Long-term consistency of individual differences would strongly support the idea that reactivity of dairy cows to challenges is mediated by stable underlying temperamental characteristics.

In addition, with the aim to further understand and validate putative temperamental characteristics in dairy cows, associations between behavioural and physiological measures were systematically examined, and a pharmacological agent was used to study the biological meaning of the reactions of dairy cattle to open field and novel object tests. Multivariate correlational analyses were employed throughout the thesis to identify patterns of intercorrelations between multiple behavioural and physiological measures. Importantly, no *a priori* assumptions were made about the level of aggregation of different behavioural and/or physiological measures (for example, in terms of traits, personality types, or behavioural syndromes, see Erhard and Schouten, 2001; Sih et al., 2004a, b), or about the nature of underlying temperamental characteristics (for example, in terms of fearfulness or coping style, see Boissy and Bouissou, 1995; Hopster, 1998). Thus, in the current thesis, terms like “trait”, “dimension”, or “characteristic” are all used interchangeably, and refer to a common entity or predisposition of unknown complexity or scale.

Finally, the research reported in the present thesis aimed to study individual differences in dairy cows’ responses to a “real life” challenge, and to correlate these differences to temperamental dimensions observed in the same animals during rearing.

Outline of the thesis

In chapter 2, individual differences in behavioural responses of dairy calves to open field, novel object, response to human and restraint tests were investigated. Variability of responsiveness of calves to open field and novel object tests was examined in more detail in chapter 3 by including physiological and neuroendocrine measures reflecting autonomic nervous system and hypothalamo-pituitary-adrenocortical (HPA) axis responses. Chapter 4 describes a pharmacological validation experiment investigating the effects of the fear-reducing agent brotizolam on the behavioural and physiological responsiveness of calves to a combined open field and novel object test. Being machine milked for the first time is a novel and potentially stressful experience for dairy heifers. Therefore, consistency of individual differences in behavioural and physiological responses of heifers to first-time machine milking was the subject of

research described in chapter 5, based on the assumption that underlying temperamental characteristics might play a role here. This assumption was further explored in a study reported in chapter 6, on the relationship between temperamental dimensions recorded during rearing and adult reactivity to milking. Chapter 6 also addressed the long-term consistency of behavioural and physiological responses of dairy cattle to open field and novel object tests. Finally, in chapter 7, the results of the various studies described in the present thesis are discussed in a wider context, with emphasis on current theories of individual differences in animals and man, and on the practical implications of the current findings.

CHAPTER 2

Behavioural reactivity of heifer calves in potentially alarming test situations: a multivariate and correlational analysis

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ABSTRACT

The aim of the present study was to determine the degree of consistency of individual differences in behavioural responses of heifer calves over time and across different situations. Twenty-five Holstein Friesian heifer calves were individually subjected to the same set of four behavioural tests at 3, 16 and 29 weeks of age. The tests involved measuring the calves' responses to a stationary human, a novel object, an open field (novel environment), and to tethering restraint. Principal component analysis (PCA) of measures recorded at each age was used to identify independent dimensions underlying the calves' reactivity to these situations. The consistency of individual differences was established by ranking the behavioural scores and the scores of factors extracted by PCA and then estimating the correlations between the ranks of each individual calf at each of the test ages. At each age, PCA revealed four factors that could be labelled: "Locomotion", "Vocalization", "Interaction with a novel object", and "Interaction with a human". Individual differences in 7 of 13 behavioural measures and in scores of all factors except "Interaction with a human" were consistent between 16 and 29 weeks of age. Rank orders for scores of the factor labelled "Interaction with a novel object" were significantly correlated across all three ages. Our results support the existence in calves of stable characteristics mediating reactivity to challenge. Multiple behavioural dimensions obtained with PCA suggest that behavioural responsiveness of calves to challenge is governed by a number of underlying factors rather than univariate phenomena, such as fearfulness or coping style.

Keywords: Calf, Individual differences, Fear, Coping, Principal component analysis

INTRODUCTION

The notion that individual differences in behavioural responsiveness of animals to stressful stimuli may be governed by stable biological characteristics (or traits) rather than simply reflect random variation has been widely recognized. These individual differences have often been interpreted in terms of either coping style or fearfulness, both of which are assumed to be mediated by underlying biological (including genetic) properties of the individual animal (Jones, 1996; Koolhaas et al., 1999). The concept of coping style emphasizes that individuals exhibit qualitative differences in their behavioural and physiological response patterns when challenged and that they may adopt either a passive or a more active coping strategy. For example, a passive coper would show inactivity, silence and an elevated adrenocortical response to challenge whereas active copers would be characterised by struggling, fighting, calling, locomotion and an enhanced catecholamine response (Benus et al., 1991; Jones and Satterlee, 1996; Koolhaas et al., 1997). Fear and fearfulness represent other cornerstones of current thinking about the biological basis of individual differences in behaviour. Fear has been defined in many ways but there is reasonable consensus that it can be best viewed as an internal, adaptive emotional state that is induced by the perception of actual danger and that fear behaviour functions to protect the animal from injury (Boissy, 1995; Jones, 1996). Fearfulness is the underlying constitutional trait that determines the propensity of an individual to be more or less frightened, (i.e., to experience an emotional state of fear), and to show fear responses in potentially alarming situations (Lyons, 1992; Boissy, 1995; Jones, 1996). Although several fear behaviours have been defined, unlike the concept of coping style, those of fear and fearfulness do not necessarily presuppose that an animal will engage in a prescribed type of behavioural response, e.g., active or passive, when it perceives a fear-eliciting stimulus, (Boissy, 1995; Jones, 1996).

From an empirical point of view, similar data is often used to support the concepts of coping style and fearfulness. It has been argued that evidence supporting the concept of coping style should entail consistency of individual differences over time and across a range of different contexts (Jensen, 1995; Koolhaas et al., 1999). Likewise, animals with a predisposition to be easily frightened are expected to show more pronounced fear responses in a wide variety of challenging situations than their less fearful counterparts (Boissy, 1995; Jones, 1996). Indeed, different authors using similar experimental challenges have interpreted cross-situational consistency in terms of both coping style and underlying fearfulness. For example, Hessing et al. (1994) claimed that the

association between the responses of individual pigs in a “backtest” (struggling during manual inversion and restraint) and their behavioural reactions to novelty supported the existence of distinct coping styles in pigs. Thus, pigs that approached a novel object quickly or reluctantly were categorised as active or passive copers, respectively (Hessing et al., 1994). On the other hand, the latency to approach a novel object has been used as a “reference measurement of fear” in poultry, pigs and cattle, with long latencies putatively indicating high levels of fear and vice versa, and the correlations between this measure and scores in other frightening test situations were thought to reflect control by the same underlying variable, i.e. fearfulness. (Boissy and Bouissou, 1995; Hemsworth et al., 1996; Jones, 1996).

Studies of individual differences in the behavioural reactivity of farm animals have not only differed in the theoretical construct used to interpret the consistency of such differences but also in the extent to which it was actually revealed across different contexts. Reports of consistency of individual differences across several different situations in a number of avian and mammalian species (Jones, 1987a; Jones and Waddington, 1992; Hessing et al., 1994; Boissy and Bouissou, 1995; Le Scolan et al., 1997; Thodberg et al., 1999) support the hypothesis that the responsiveness of animals to environmental challenge could be mediated by a single underlying characteristic. In this respect, terms like “general reactivity” (e.g., Thodberg et al., 1999), “temperament” (Goldsmith et al., 1987) or “general fearfulness” (e.g., Goddard and Beilharz, 1984; Jones, 1987a; Vandenheede et al., 1998) have been used to signify a major unifying dimension (or trait) on which multiple behavioural variables are distributed. However, other studies established consistency of individual differences across only a relatively limited number of similar situations (Lawrence et al., 1991; Pollard et al., 1994; Jensen et al., 1995a; Spoolder et al., 1996). Alternative frameworks that have been proposed to interpret individual differences in reactivity may account for such apparent inconsistencies. For example, Ramos and Mormède (1998) consider fearfulness (labelled as “emotionality”) to be multidimensional and to encompass different forms of fear responding, involving different emotional states, that may be independently displayed under different conditions in different animals. Thus, an animal that is fearful in one situation may not experience an emotional state of fear in another situation. This idea resembles Wilson’s (1998) notion of “domain-specificity”, meaning that an animal may express one or another behavioural disposition (e.g., “shyness” or “boldness”), depending on the environmental “domain” or context.

Increased understanding of the origins and implications of individual differences is extremely important because it may enable: i) the prediction of behaviour responses in one situation based on those expressed in another and/or ii) the (early) identification of animals that may have life-long difficulty in adapting to challenge (Boissy, 1995; Spoolder et al., 1996; Koolhaas et al., 1999).

The present experiment was designed to study individual differences in behavioural responsiveness to varying challenges in dairy calves. Our objective was to simultaneously investigate the consistency of individual differences over time and across different situations. Therefore, heifer calves were repeatedly subjected to the same set of potentially alarming situations at 3, 16 and 29 weeks of age. We reasoned that long-term consistency of individual differences should be demonstrated if calf reactivity is mediated by individually stable characteristics. The tests measured the calves' responses to a stationary human being, a novel object, an open field (novel environment) and restraint, respectively. Avoidance responses of cattle in the 'human' and the 'novel object' tests are thought to reflect fear of people (Tilbrook et al., 1989) and of novelty (Boissy and Bouissou, 1995), respectively. Reactivity in the open field and the restraint tests was expected to reveal elements of activity (e.g., locomotion) and physical resistance, which have been implicated in the distinction between an active and a passive coping style (Erhard et al., 1999; Ruis et al., 2001). We employed principal component analyses (PCA) to examine the extent to which variation in the responsiveness of the calves could be attributed to a single underlying dimension, e.g., fearfulness or coping style.

MATERIALS AND METHODS

The present experiment was carried out at the experimental farms of the Animal Sciences Group of Wageningen UR, The Netherlands. The experiment was approved by the Institute's Animal Care and Use Committee.

Animals, housing and management

Twenty-five Holstein Friesian heifer calves were used. All animals were born at the same experimental farm of ID-Lelystad, The Netherlands, in the same calving season (autumn). They were healthy and had a birthweight equal to, or exceeding 40 kg. Five groups (batches) of five calves each were established according to date of birth at a regular rate of one group every three weeks. Within groups, the ages of the individual calves differed by no more than 3 days from the group mean.

Following birth, calves were immediately separated from the dam and housed individually in straw-littered pens. They were fed colostrum for the first 2 days post-partum; this was replaced by whole milk given in gradually increasing daily amounts during rearing to a maximum of 8 l/calf/day. At approximately one week of age, each group of five calves was transported to another experimental farm (50 min drive), and housed together in a straw-littered pen (3 x 3 m). They remained socially housed indoors with the same penmates for the duration of the experiment. At this time the diet was supplemented with concentrates and ad libitum roughage in the form of hay and maize silage. Milk and concentrates were fed daily at 07.00 and 17.00 h by the stockman, and water was available from automatic dispensers. All calves were dehorned at 8 weeks of age. Milk supply was reduced to 50% of the maximum allowance at 10 weeks and the calves were fully weaned at 11 weeks of age. At 13 weeks of age, (10 weeks after the first series of behavioural tests, see below), the calves were transferred back to the original farm of birth and group-housed on straw. Five group pens adjacent to each other, and thereby allowing visual and tactile contact between neighbouring groups, accommodated the 25 calves. Additional behavioural testing took place at this farm. After completion of the final set of behavioural tests, the groups of heifers were moved successively onto pasture.

Behavioural tests

Each calf was individually subjected to the same series of four behavioural tests at the ages of 3, 16 and 29 weeks. At each age, calves of the same group were tested

on four consecutive days with one of four behavioural tests conducted on each day. The order of testing within ages was: the stationary human, open field, novel object and restraint tests. The calves were tested in a random order each day. All testing took place in large, enclosed rooms separate from the home pens and containing an arena or a designated testing area. The walls of test arenas were made of pre-fabricated, 2 m high wooden panels. The dimensions of the arenas increased with age (see below) in an attempt to maintain approximate constancy over time within the same test in the ratio between the average size of the calves and the surface area of the arena. Average sizes in terms of square metres occupied per animal were estimated to be 0.40, 0.70 and 0.95 at 3, 16 and 29 weeks, respectively (estimates based on the estimated relationships between size and average weight in young cattle of 50, 110 and 200 kg, which approximates to average weights of our heifer calves at 3, 16 and 29 weeks of age, respectively).

Calves were individually transported to and from the testing room in a wheeled cart. The distance between the home pens and the test arenas approximated 40 and 20 m at the first and second experimental farm, respectively. To facilitate handling, calves were fitted with halters one week before each episode of testing. In the week prior to testing calves were led with the halter and briefly transported in the cart on three occasions in order to habituate them to the testing conditions. Calves were not exposed to this room prior to testing.

Apart from the open field, all tests were carried out on a floor littered with a layer of approximately 15 cm straw. After each test any faeces or urine was removed, a little fresh straw was added, and the entire bedding material was redistributed evenly. All the straw was completely refreshed before testing at the next age band began. Open field tests were conducted on a solid concrete floor which was sufficiently rough to provide adequate footing, i.e. to prevent slipping. This floor and that of the connecting start box (see below) were cleaned with a high pressure hose and excess water was removed with a scraper before each calf was tested.

All behavioural tests were recorded onto videotape using cameras fitted to the ceiling. A microphone was used to record vocalizations. Tests were also observed directly via a monitor situated outside the test room. Behavioural measures were analysed using the Observer Software System for Behavioral Research (Noldus Information Technology, Wageningen, The Netherlands) and part of this software system was downloaded onto a portable computer for direct observations (see below). Definitions of each behavioural measure are given in Table 2.1.

Response to human test. Here, we adopted a protocol that has been widely used for recording the behavioural reactions of individual cattle to a stationary person (Tilbrook et al., 1989; Hemsworth et al., 1993, 1996). We used a straw-littered rectangular test arena with a manually operated slide door in the corner of one of the (shorter) walls. Dimensions of the arena were 4 x 3 m, 5 x 4 m, and 6 x 4.5 m, as the calves were tested at 3, 16 and 29 weeks, respectively. Calves were individually introduced into the test arena for a 3-min familiarization period. A female experimenter (the same person throughout the entire study) then quietly entered the arena and assumed a stationary position at the midpoint of the wall opposite the entrance and facing the centre of the arena. She always wore white overalls, in contrast to the green ones normally worn by the barn staff. During the next 10 min the following measures were recorded: the latencies for each calf to approach within 1 m of the human and to make the first physical contact with her, the accumulated times spent in contact with the experimenter and in locomotion, and the number of vocalizations (see Table 2.1). The experimenter operated a portable computer during testing and recorded whether a calf touched her or not. Other measures were made by analyses of the videotapes.

Open field test. Our procedure resembled that used in many other studies of bovines (Kilgour, 1975; Boissy and Bouissou, 1995; Jensen et al., 1997). Calves were individually confined in a start box for 3 min. This was constructed so that the calf could not turn around inside it or see out of it and it was connected to the test arena by a manually operated slide door in the middle of one wall of the square arena. Dimensions of the arena were 4 x 4 m, 4.5 x 4.5 m, and 6 x 6 m, for 3, 16 and 29 week-old-calves, respectively. After the door was opened, calves could enter the arena. However, rather than allowing each calf a prescribed amount of time (e.g., one min) to voluntarily enter the arena and then pushing in those animals that had not left the start box by this time (e.g., Boissy and Bouissou, 1995; Jensen et al., 1997), in the present study the experimenter briefly touched the lower back of each calf immediately after opening the door so as to ensure that all calves entered the arena at the same time and in a similar way. We reasoned that pushing some animals and allowing others to enter voluntarily could bias the test. Behaviour was recorded for 10 min after each calf entered the arena. Behavioural measures included the accumulated times spent in locomotion and in contact (with nose or tongue) with the floor or the walls, and the number of vocalizations (see Table 2.1).

Table 2.1

Definition of behavioural measures recorded in heifer calves during various tests at the ages of 3, 16 and 29 weeks

Measures	Definition
Recorded during multiple tests[a]	
Locomotion	Movement of front legs or all four legs
Vocalization	All types of vocalizations
Response to Human Test	
Latency to approach within 1 m of the human	Time to enter a radius of 1 m from the human with both front legs
Latency to first contact with the human	Time until the first contact with the human with nose or tongue
In contact with the human	Sniffing, touching or rubbing the human with nose, tongue or head
Open field test	
In contact with floor/walls	Sniffing or touching the floor or walls with nose or tongue
Novel object test	
Latency to approach within 1 m of the object	Time to enter a radius of 1 m from the object with both front legs
Latency to first contact with the object	Time until the first contact with the object with nose or tongue
In contact with the object	Sniffing, touching or rubbing the object with nose, tongue or head
Restraint test	
Movement of head or legs	All such movement
Rope stretched	Rope is stretched tight between halter and wall

[a]Locomotion and vocalization were recorded during the Human test, during the Open field test, and during the Novel object test

Novel object test. Procedures in this test resembled those described by Lawrence et al. (1991) for pigs. Calves were individually introduced into a straw-littered rectangular test arena with a manually operated slide door in the corner of one of the (shorter) walls. Dimensions of the arena were 3.5 x 2 m, 4.5 x 3 m, and 5 x 4 m, at 3, 16 and 29 weeks, respectively. After a 3-min familiarization period, a novel object connected to a rope was lowered rapidly from the ceiling to the floor at 1 m from the centre of the arena and directly opposite the midpoint of the wall facing the entrance. The novel object consisted of a tambourine attached to a coloured plastic ball (30 cm diameter). It was sufficiently weighted to create a soft noise when it hit the floor, thereby attracting the attention of each calf, and it was then immediately pulled back up to a height of 0.5 m and left in that position for the 10 min test. The following behaviours were recorded: the latencies for each calf to approach within 1 m of the object and to make physical contact with it, the accumulated times spent in contact with the object and in locomotion, and the number of vocalizations (see Table 2.1).

Restraint test. The restraint test was carried out in an area of the test room (> 30 m2 at all ages) delineated at one end by the wooden wall of one of the previously described test arenas located inside the room and at the other by a stone wall. Calves were individually tethered for the 10 min test with a rope connecting the halter to a metal ring attached to the stone wall. The length of the rope and the height of the metal ring varied as the calves grew. Thus, the rope measured 0.65 m, 0.80 m, and 1.0 m, and the metal ring was situated at a height of 0.55 m., 0.70 m and 0.90 m off the floor, when the calves were 3, 16 and 29 weeks old, respectively. Our behavioural measures were the accumulated times when the calf was moving its head or legs and when it had moved away sufficiently from the wall for the rope to be stretched tight (see Table 2.1).

Statistical analyses

The times spent in locomotion, in contact with the floor or walls of the open field, in contact with the human or the novel object, moving the head or legs, and when the rope was stretched were all expressed as percentages of the total test duration.

Preliminary analyses revealed that at all three ages the latencies to first contact with the human and the novel object were strongly correlated with the latencies to approach within 1 m of both stimuli; Spearman rank correlations (Conover, 1980) were always greater than 0.75 ($P < 0.001$) apart from the 3-week novel object test when this correlation was 0.46 ($P < 0.05$). Therefore, in subsequent analyses we focused on the latencies to first contact rather than on the approach latencies.

All analyses were conducted using Genstat (Genstat Committee, 2000).

Analyses for group effects. Potential effects of groups (i.e. batches) were examined at each test age. The percentages of test time were analysed as fractions with a logistic regression model, comprising a multiplicative overdispersion factor with respect to the binomial variance function. The number of vocalisations, i.e., count data, were analysed using a log linear model comprising a multiplicative dispersion factor relative to the Poisson variance function. Latencies were log transformed (log(y+1), and unity was added to accommodate y = 0, (which occurred in a number of response to human tests) prior to analysis of variance. Latencies that reached the upper limit were entered as censored observations (Taylor, 1973) but this hardly affected the results so it was concluded that censored latencies offered no problems for analysis.

In all models, groups were entered as levels of an experimental factor. Analyses of logistic and log linear models were based on maximum quasi likelihood with overdispersion parameters estimated from Pearson's generalized chi-square statistic (McCullagh and Nelder, 1989). No significant differences (F-test in analysis of variance and quasi likelihood ratio test in logistic and log linear models) were found between groups. Therefore group was not included as a factor in subsequent analyses.

Analysis of differences between tests or between ages within animals The levels of measures recorded within animals in the different tests were compared within ages as well as within the same test at different ages, using the Wilcoxon matched pairs signed rank test (Conover, 1980). Tests on differences were performed to avoid the need for modelling complex dependence structures between data from the same animal.

Analysis of correlation structure. Principal component analysis (PCA) (Jolliffe, 1986) was used to investigate the structure of the correlation matrix within ages and to condense correlated measures into so-called principal components. These are linear combinations of the original measures, reflecting independent characteristics (or dimensions) underlying the correlation matrix. The loading of each measure on a principal component represents the correlation between the latent characteristic and the original measure and thus indicates the importance of a measure for a principal component. If the reactivity of animals to environmental challenge was mediated by a single underlying trait, PCA of measures recorded in different situations would be expected to produce at least one major component (i.e., underlying dimension) with high loadings from multiple measures (e.g., Goddard and Beilharz, 1984). Eight behavioural measures obtained in three different tests were included in a PCA: the latencies to first contact with the human and with the novel object, the times spent in contact with each of

these stimuli, and locomotion and vocalization during each of the open field and novel object tests. We included these latter variables in a PCA since for each of them it has been argued that, in cattle, individual differences may reflect differences in the same underlying characteristic, e.g. temperament or fearfulness (Boissy and Bouissou, 1995; De Passillé et al., 1995; Hemsworth et al., 1996; Grignard et al., 2000. See also the discussion of the present paper).

A separate PCA was carried out on the data collected at each age. After extraction, principal components were scaled by their standard deviations (square roots of associated eigenvalues) and subjected to varimax rotation. Scaling ensures that the factors obtained after rotation are independent, like the principal components prior to scaling

Prior to PCA, latencies and vocalisations were log transformed (log(y+1)) and fractions of time were logit transformed (log(y/(1-y), replacing y = 0 by y = 0.1 * minimum of positive fractions). Transformation ensures that the distributions of respective variables are close to multivariate normality and, correspondingly, that associations between variables are best described by linear functions.

Relation between measures. Spearman rank correlations were calculated for pairs of measures, including scores of factors derived from PCA at different ages, in order to determine the relationships between them and to establish whether individual differences were consistent over time.

Correlations between multiple factor scores obtained at different ages were analysed using canonical correlation analyses (CCA) (Mardia et al., 1979), and stepwise multiple regression. The aim of CCA is to concisely describe the dependence structure between two sets of multiple measures. The first canonical variables of each set are linear combinations of the original measures that have the highest correlation of all possible linear combinations. The highest correlation is referred to as the canonical correlation.

RESULTS

Differences in average levels of measures between ages and between tests

Means and standard errors of the behavioural responses measured during the four tests at each age are presented in Table 2.2.

Table 2.2

Behavioural measures (mean ± S.E.) recorded in heifer calves during each test at 3, 16 and 29 weeks of age

	Age					
	3 Weeks		16 Weeks		29 Weeks	
Measure	Mean	S.E.	Mean	S.E.	Mean	S.E.
Response to human test						
Locomotion (% of time)	8.9^{a,p,q}	1.22	8.2^{a,p}	1.09	12.7^{b,p}	1.12
In contact with the human (% of time)	4.6^{a}	0.81	13.4^{b}	2.42	18.6^{b}	2.59
Latency to first contact with the human (s)	180.0^{a}	47.31	92.2^{a,b}	28.73	45.7^{b}	22.70
Vocalizations (number)	1.4^{a,b,x}	0.48	1.6^{a,x}	0.46	2.9^{b,x}	0.74
Open field test						
Locomotion (% of time)	11.5^{a,p}	1.58	19.7^{b,q}	1.29	25.4^{c,q}	1.60
In contact with floor/walls (% of time)	7.6^{a}	1.50	17.4^{b}	1.92	18.1^{b}	1.83
Vocalizations (number)	1.1^{a,x}	0.41	14.1^{b,y}	2.75	12.5^{b,y}	2.20
Novel object test						
Locomotion (% of time)	6.5^{a,q}	0.88	10.0^{b,p}	0.98	16.3^{c,p}	1.50
In contact with the object (% of time)	4.4	1.32	5.0	1.35	3.6	0.81
Latency to first contact with the object (s)	271.6^{a}	45.99	223.0^{a}	41.22	86.3^{b}	26.27
Vocalizations (number)	0.1^{a,y}	0.04	6.1^{b,z}	1.71	6.9^{b,x}	1.64
Restraint test						
Movement of head or legs (% of time)	26.6^{a,b}	2.86	29.3^{a}	2.34	20.6^{b}	1.92
Rope stretched (% of time)	53.8^{a}	4.25	29.6^{b}	3.38	16.5^{c}	2.64

S.E. = standard error, % = percentage, (s) = seconds

^{a,b,c}Different superscripts within a row indicate a significant difference ($P < 0.05$) between ages

^{p,q}Different superscripts within a column indicate a significant difference ($P < 0.05$) in locomotion between tests

^{x,y,z}Different superscripts within a column indicate a significant ($P < 0.05$) difference in vocalizations between tests

The times spent in locomotion during the human, open field and novel object tests increased significantly with age. At 16 and 29 weeks of age, locomotion was more pronounced in the open field than in the response to human and the novel object tests. At 3 weeks, vocalization was relatively infrequent in all tests (mean < 1.5 vocalizations per 10-min test) and it remained at a similar level in the human test. In contrast, calves exhibited a marked increase in the number of vocalizations during the open field test and, to a lesser degree, during the novel object test at 16 and 29 weeks. Time spent in contact with the novel object remained constant across test ages, but contact with the human was greater at 16 and 29 weeks than at 3 weeks. In the restraint test, the accumulated time when the rope was stretched decreased markedly with age.

Correlational analyses

Principal Component Analysis. PCA, with orthogonal rotation, of eight behavioural measures revealed a multifactorial picture at all ages (Table 2.3). Eigenvalues of the first three factors at 3 and 16 weeks and of the first four factors at 29 weeks were greater than 1, indicating that multiple underlying factors contributed to the percentage of variance accounted for. Cumulative percentages of total variation explained by the first four factors were similar across age: 81%, 83%, and 83% at 3, 16 and 29 weeks, respectively. Different measures separated out well on different factors, i.e., the same measure generally carried a high loading on one factor only. Loading patterns at 16 and 29 weeks were virtually identical, except that the order of factors according to eigenvalue differed slightly. One factor had high loadings for vocalization during the open field and the novel object tests whereas another had high loadings for locomotion during these two tests. Behavioural measures related to the calves' interaction with a human and with the novel object (i.e., latency to first contact and time spent in contact) loaded highly on the third and fourth factors. Thus, at 16 and 29 weeks, different factors could be labelled in a straightforward and similar manner, based on the importance of measures defined by loadings (Table 2.4). The pattern of loadings at 3 weeks allowed for tentative labelling of factors in line with the other ages, although Factors 2 and 3 also involved high loadings for behavioural measures not reflected in the label (see Table 2.3 and Table 2.4).

Table 2.3

Loadings[a] on the first four factors extracted by principal component analyses (PCA), after varimax rotation, of behavioral measures recorded in heifer calves at the ages of 3, 16 and 29 weeks, and the eigenvalues and proportions of total variation explained by each factor

	Age											
	3 Weeks				16 Weeks				29 Weeks			
Measures	Factor 1	Factor 2	Factor 3	Factor 4	Factor 1	Factor 2	Factor 3	Factor 4	Factor 1	Factor 2	Factor 3	Factor 4
In contact with human (% of time)	**0.83**	0.31	- 0.15	0.25	- 0.23	0.40	- 0.02	**0.82**	- 0.14	0.02	0.02	**0.95**
Latency to first contact with human (s)	**- 0.92**	0.10	- 0.02	- 0.04	- 0.01	0.22	- 0.15	**- 0.88**	0.14	- 0.06	0.02	- 0.18
Locomotion during OFT[b] (% of time)	0.21	0.02	0.01	**0.94**	0.01	- 0.06	**0.97**	0.01	0.02	**0.91**	- 0.01	0.18
Vocalizations during OFT (number)	0.18	0.05	- 0.03	0.14	**- 0.90**	- 0.13	0.04	0.09	**- 0.93**	0.15	- 0.00	0.11
Locomotion during NOT (% of time)	- 0.13	**0.57**	**- 0.56**	0.36	- 0.30	0.24	**0.84**	0.16	- 0.03	**0.92**	- 0.18	- 0.11
In contact with object (% of time)	- 0.04	**0.95**	0.06	- 0.18	0.22	**0.87**	0.10	0.03	0.01	- 0.05	**0.94**	0.10
Latency to first contact with object (s)	- 0.32	**- 0.80**	- 0.12	- 0.26	0.02	- 0.25	0.13	- 0.14	- 0.24	0.28	**- 0.63**	0.45
Vocalizations during NOT (number)	0.11	- 0.16	**- 0.92**	- 0.07	**- 0.90**	- 0.05	0.14	0.07	**- 0.93**	- 0.14	- 0.11	0.10
Eigenvalues	2.61	1.75	1.21	0.87	2.40	1.76	1.48	0.93	2.59	1.79	1.17	1.01
Variance explained	33%	22%	15%	11%	30%	22%	19%	12%	32%	22%	15%	13%

% = percentage, (s) = seconds

[a]Loadings greater than 0.50 are indicated in bold

[b]Abbreviations of behavioural tests: OFT = Open field test; NOT = Novel object test

Table 2.4

Tentative labels for the first four factors extracted by principal component analyses (PCA), after varimax rotation, of test measures recorded in heifer calves at 3, 16 and 29 weeks of age[a]

	Age		
Factor	3 Weeks	16 Weeks	29 Weeks
1	Interaction with a human	Vocalization	Vocalization
2	*Interaction with a novel object*[b]	Interaction with a novel object	Locomotion
3	*Vocalization*	Locomotion	Interaction with a novel object
4	Locomotion	Interaction with a human	Interaction with a human

[a]See Table 2.3 for loadings of measures on factors

[b]Labels indicated in italics refer to factors with loading patterns that also involve high loadings of measures other than those reflected in the label

Consistency of measures over time. Individual differences in 7 of the 13 measures were consistent over time between 16 and 29 weeks of age (Table 2.5). However, for most measures, individual differences at 3 weeks did not reliably predict those at 16 and 29 weeks. Behavioural responses during the human test correlated poorly across ages in contrast to those recorded in the other tests. Long-term consistency of individual differences (from 3 to 29 weeks) was found for 3 measures; these were: locomotion in the open field, time in contact with the novel object, and time spent moving the head or legs during restraint (Table 2.5).

Individual differences in scores of 3 of the 4 factors extracted by PCA at each age also proved to be consistent over time, particularly between 16 and 29 weeks (Table 2.5). Calves were consistent across age in their tendency to show high or low levels of locomotion or vocalization in different situations (i.e., the open field and the novel object tests). Individual differences in scores of the factor labelled “Interaction with a human” were not consistent over time, whereas rank orders for scores of the factor labelled “Interaction with a novel object” were significantly correlated across all three test ages (Table 2.5).

Table 2.5

Spearman rank correlations between test measures recorded at 3, 16 and 29 weeks in heifer calves, and between scores of factors[a] extracted by principal component analyses (PCA)

	Interval between ages		
Measures	3 - 16 weeks	3 - 29 weeks	16 - 29 weeks
Rsponse to human test			
Locomotion (% of time)	0.31	0.13	0.18
In contact with the human (% of time)	- 0.09	- 0.13	0.28
Latency to first contact with the human (s)	- 0.19	- 0.07	0.23
Vocalizations (number)	- 0.14	0.05	0.67**
Open field test			
Locomotion (% of time)	0.45*	0.62**	0.76***
In contact with floor/walls (% of time)	0.17	0.21	0.53*
Vocalizations (number)	0.20	0.26	0.88***
Novel object test			
Locomotion (% of time)	0.14	0.15	0.59**
In contact with the object (% of time)	0.60**	0.65**	0.59**
Latency to first contact with the object (s)	0.17	0.01	0.31
Vocalizations (number)	0.35	0.26	0.77***
Restraint test			
Movement of head or legs (% of time)	0.17	0.44*	0.18
Rope stretched (% of time)	0.05	- 0.42*	0.20
Factors			
Locomotion[b]	0.31	0.42*	0.67***
Vocalization	- 0.05	- 0.11	0.86***
Interaction with a novel object	0.45*	0.57**	0.72***
Interaction with a human	- 0.24	- 0.22	0.08

% = percentage, (s) = seconds

* P <0.05; ** P <0.01; *** P <0.001

[a]See Table 2.3 for loadings of measures on factors

[b]See Table 2.4 for labels of factors at each age

Correlations between measures and multiple regression. We analysed rank correlations between scores of PCA factors and behavioural measures that were not included in the PCA to examine the relationships between the latter and the underlying dimensions of the data. At 29 weeks, but not at 3 or 16 weeks, locomotion during the human test and the time spent moving during restraint were positively correlated with scores of the factor labelled "Locomotion" (rank correlations 0.43 and 0.48, respectively, $P < 0.05$). Given positive loadings on this factor for locomotion during the open field and novel object tests (Table 2.3), this finding suggests inter-situational consistency of locomotion/movement scores across all four behavioural tests at 29 weeks of age. Analysis of vocalisation revealed no significant correlations at any age.

Although the PCA revealed no correlation within ages between the responses of calves to a human and to a novel object, (in terms of latencies to first contact and times spent in contact), additional analyses pointed to a more subtle link between reactivity to a human and to a novel object at the age of three weeks only. Canonical correlation analyses and stepwise multiple regression on scores of factors extracted by PCA indicated that the factor labelled "Interaction with a novel object" at 16 weeks was best predicted by a combination of factors labelled "Interaction with a novel object" and "Interaction with a human" at 3 weeks of age. Coefficients of canonical variates conferring the highest canonical correlation between the ages of three and 16 weeks are given in Table 2.6.

Table 2.6

Canonical variate coefficients[a] for factors[b] extracted by PCA from calves' responses at 3 and 16 weeks of age

	Age	
Factors	3 weeks	16 weeks
Interaction with a human[c]	0.11	- 0.08
Interaction with a novel object	0.14	0.17
Vocalization	0.06	0.05
Locomotion	0.09	0.04

[a]Canonical orrelation between canonical variates at the ages of 3 and 16 weeks is 0.68

[b]See Table 2.3 for loadings of measures on factors

[c]See Table 2.4 for labels of factors at each age

A high coefficient at 16 weeks for “Interaction with a novel object” (0.17) was associated with relatively high coefficients (exceeding 0.10) for “Interaction with a novel object” and “Interaction with a human” at 3 weeks (Table 2.6). Correspondingly, the best fit (i.e., the highest percentage variance accounted for) after regression analyses using scores of the factor labelled “Interaction with a novel object” at the age of 16 weeks as the dependent variable and scores of all four factors extracted at the age of three weeks as explanatory variables, was obtained by a model with factors labelled “Interaction with a novel object”, “Interaction with a human”, and “Locomotion” as covariates (regression coefficients and P-values of covariates: 0.42 and 0.03, 0.33 and 0.08, 0.23 and 0.22, respectively). These findings provide support for the suggestion that the behavioural response of calves to a human at the age of 3 weeks may be controlled by the same underlying process as their responsiveness to a novel object.

DISCUSSION

The present experiment investigated the consistency of individual differences in the responsiveness of heifer calves across four intuitively alarming test situations and across three different ages (3, 16 and 29 weeks). With the help of PCA, cross-situational consistency was analysed within each age in order to identify independent dimensions underlying reactivity. The main hypothesis tested in this respect was that responsiveness to challenge in calves is mediated by a single characteristic, such as fearfulness or coping style. Consistency of individual differences over time was evaluated by correlating the rank orders of behavioural measures and of scores of factors extracted by PCA across ages.

PCA revealed a multifactorial picture at all ages. Moreover, individual behavioural measures mainly loaded onto one factor only (or, in the case of measures not included in the PCA, correlated with scores of one factor only); this suggests a relative lack of inter-correlation between many of the measures recorded in the present study. Thus, our results do not support the hypothesis that the behavioural responses of calves to several different challenging situations are controlled by a single underlying mechanism or trait. In the present experiment, four mutually uncorrelated sets of behavioural measures were identified, reflected in four orthogonal factors obtained with PCA. These were: locomotion, vocalization, interaction with a novel object, and interaction with a human. In previous studies it had been proposed that such sets of behaviours were linked to the same underlying characteristic. For example, work with (dairy) cattle suggested that individual differences in latencies to approach humans or objects as well as differences

in locomotion and vocalization during novel object, response to human, and open field tests were all associated with differences in fearfulness (Boissy and Bouissou, 1995; De Passillé et al., 1995; Hemsworth et al., 1996; Grignard et al., 2000). Further, in studies with pigs, individual differences in locomotion and vocalization during open field and novel object tests and in latencies to approach a human or a novel object have been assumed to reflect differences in coping style (Hessing et al., 1994; Ruis et al., 2000, 2001). However, the present findings are not consistent with an interpretation of individual differences in terms of a univariate phenomenon. Therefore, we now consider a number of alternative interpretations.

First, the pattern of responsiveness in our calves may have reflected the existence of multiple traits that independently mediated behavioural reactivity. This notion is consistent with previous suggestions that different tests, or different aspects of the same test (e.g., novelty, social isolation), may measure different types of fearfulness or "emotionality" corresponding to different emotional dimensions (e.g., Griebel et al., 1996; Ramos and Mormède, 1998; Wilson, 1998; File, 2001). Extrapolated to the present data, this interpretation would imply that different emotional states varied independently among calves and that they were differentially expressed, e.g., by vocalization, by locomotion, or by approach/avoidance responses towards a novel object or a human.

A slightly modified explanation is that traits other than fearfulness also mediate reactivity to alarming situations. For example, it has been argued that behaviours exhibited by calves in an open field test may reflect locomotor or exploratory tendencies as well as fearfulness (Munksgaard and Jensen, 1996; Jensen et al., 1997; Rushen, 2000). Thus, one set of behavioural responses recorded in our calves may have been predominantly controlled by underlying fearfulness and/or curiousity, e.g., those related to interaction with a novel object (Salzen, 1979; Boissy and Bouissou, 1995; Jones, 1996), whereas locomotion or vocalization may have been regulated by other independent predispositions. A likely candidate trait is "sociality", i.e., the extent to which animals need social companionship (Jones and Mills, 1999; Erhard and Schouten, 2001). The rate of vocalization during social isolation of a previously group-housed animal is believed to constitute an important behavioural correlate of this trait in bovines, sheep and poultry (Jones and Mills, 1999; Jones et al., 1999; Watts and Stookey, 2000; Erhard and Schouten, 2001). For example, lines of quail have been selected for high or low levels of "social reinstatement behaviour" (a measure of sociality), independently of selection for long or short durations of tonic immobility (a measure of fearfulness), thus suggesting that sociality and fearfulness may be independent traits (Mills and Faure,

1991). In agreement with this suggestion, Jones et al. (1999) reported that underlying fearfulness was not a major contributory factor in determining the time taken by domestic broiler chicks to traverse a T-maze and thereby to regain visual contact with conspecifics. On the other hand, individual variation in underlying sociality was considered to be an influential variable in determining differences in T-maze perfomance (Jones et al., 1999).

In rats, locomotion in novel environments may (genetically) correlate with spontaneous activity during a 24-h cycle (Courvoisier et al., 1996; Moisan et al., 1996), and divergent selection for high or low locomotor activity in a novel environment may result in line differences in spontaneous activity in the home cage or an outdoor enclosure (Fujita et al., 1994). Thus, active behaviours exhibited by calves in tests such as those used in the present study might, at least to some extent, be mediated by "general activity", a characteristic that may not be directly linked to fearfulness (Ramos and Mormède, 1998). The suggestion that behaviours such as locomotion, vocalization and contact with a novel object may reflect different underlying biological factors not only fits the present data but also agrees with other reports of a lack of inter-correlation between vocalization, activity and other putative measures of fearfulness in farm animals. For example, the movement of cattle during isolation on a weighing scale or in a crush did not correlate with the number of vocalizations emitted (Grignard et al., 2001; Watts et al., 2001). Multivariate analyses of data obtained in pigs revealed dissociation between vocalization during social isolation and the latency to approach a novel object (Forkman et al., 1995), and between locomotion and other behaviours thought to reflect "anxiety" or "fear of novelty" (Andersen et al., 2000b).

A third interpretation of our findings, that does not necessarily exclude the previous ones, is based on the concept of coping style. This concept assumes that animals within any population will vary in their likelihood of adopting an active or a passive coping strategy when exposed to threatening or aversive events (Benus et al., 1991; Mendl and Deag, 1995; Koolhaas et al., 1999; Erhard and Schouten, 2001). Active coping is characterized by high levels of locomotion, struggling, and vocalisation whereas passive coping entails immobility and silence (Jones and Satterlee, 1996; Erhard et al., 1999; Ruis et al., 2001). It is tempting to suggest that consistent individual differences in locomotor activity found in the present study may have reflected variations in coping style, particularly because scores of the "Locomotion" factor correlated with the time spent moving the head or legs (struggling) during restraint, at least in 29-week-old calves.

Locomotion in our tests may have reflected a “general activity” trait (see above), in which case there is no need to make assumptions about underlying emotional states. Alternatively, the amount of locomotion may indicate the level of fear, with, for example, low locomotion reflecting high fear and vice versa (Boissy and Bouissou, 1995; Jones, 1996). A different line of reasoning is called for if one interprets locomotor activity in terms of coping style. For instance, an alarming situation might elicit a similar emotional state (i.e. level of fear or distress) in two calves but this may be associated with very different behavioural responses (e.g. high or low locomotion) if they differ in their coping style. In other words, passive and active copers may show qualitatively different behavioural patterns, ranging from immobility to escape, when they experience a frightening situation. Thus, the dissociation between “Locomotion” and “Interaction with a novel object” found in the present study may represent the elicitation of two different dimensions of the emotional response, associated with the behavioural strategy used when challenged and the level of fear experienced, respectively (Ramos and Mormede, 1998; Erhard et al., 1999). Similarly, two independent dimensions, namely “Emotional reactivity” (related to putative measures of fear and anxiety) and “Coping style” (locomotion, rearing) were identified following multivariate analyses of behavioural profiles in rats (Steimer et al., 1997).

An additional mechanism may underpin the lack of cross-situational consistency in our calves’ responses to the experimenter. To some extent, three-week-old calves that were reluctant to approach the novel object were also hesitant to make contact with the experimenter. A similar relationship between responses to humans and novel objects in gilts (Spoolder et al., 1996) and goats (Lyons et al., 1988) was thought to indicate consistency in underlying fearfulness (Lyons, 1992). Thus, at three weeks, calves that were frightened of the novel object may also have been fearful of the human. Fear of humans is markedly reduced by regular handling (Hemsworth et al., 1993; Jones, 1996). This phenomenon can generalize to include people other than the person who actually handled the animals (Jones, 1994; Hemsworth et al., 1996; Boivin et al., 1998). However, it may be limited to fear of humans, presumably through a process of stimulus specific habituation (Jones and Waddington, 1992; Hemsworth et al., 1993). For example, handling decreased fear of humans in chickens, beef cattle and veal calves but it did not affect their reactivity to novel stimuli (Jones and Waddington, 1992; Hemsworth et al., 1996; Lensink et al., 2000). Since the calves used here experienced frequent visual and physical contact with people, a similar mechanism involving progressive habituation to stockmen and experimenters may have operated during rearing and

repeated testing. Consequently, the 16- and 29-week-old calves observed in the response to human test may have been primarily motivated to express exploratory rather than fear behaviours (see Marchant et al., 1997). Furthermore, the experimenter may even have come to represent a source of social companionship. This might explain why calves vocalized less during the response to human than during the open field and novel object tests at 16 and 29 weeks.

The present results provide substantial support for long-term consistency of individual differences in the behavioural responsiveness of heifer calves to challenge. This consistency, particularly in scores of factors representing condensed dimensions of the data, strengthens the suggestion that such differences are indicators of basic personality traits (see Erhard and Schouten, 2001). The consistency of individual differences in locomotion and in reactivity to a novel object from 3 to 29 weeks of age demonstrates the robustness of the underlying characteristics. Our finding that individual differences remained stable over time in calves is consistent with similar observations in cats, great tits, chickens, monkeys, dairy goats and beef cattle (Verbeek et al., 1994; Jones, 1996; Durr and Smith, 1997; Kalin and Shelton, 1998; Lyons et al., 1988; Grignard et al., 2001; Watts et al., 2001).

A final note concerns developmental aspects of behaviour. Locomotion and vocalization were more pronounced in 16- and 29-week-old calves than in 3-week-old ones. Although age and test experience were confounded here, low locomotor activity and low vocalization at three weeks fit the type of behavioural strategy young bovines are thought likely to adopt in threatening situations (Kiley, 1972; Watts and Stookey, 2000). The subsequent increased consistency of individual differences between 16 and 29 weeks could be explained in terms of the stabilization of developmental processes (Hinde and Bateson, 1984; McCall, 1986). Similar increases in stability of individual differences after a certain “minimum” age have been found in dogs (Goddard and Beilharz, 1986) and children (McDevitt, 1986).

Further research is needed to determine whether and to what extent the behavioural measures recorded here reflect coping style, fearfulness, or other underlying biological characteristics. The planned examination of physiological and neuroendocrine ‘stress’ responses in conjunction with behavioural ones may be particularly relevant.

CONCLUSION

In conclusion, the present study demonstrated consistency of individual differences in the behavioural responses of heifer calves to potentially alarming events over time. This finding supports the hypothesis that the reactivity of calves to stressful situations is mediated by stable characteristics of the individual animal. However, different behavioural measures were largely uncorrelated, indicating that the behavioural responses of calves to challenge are probably mediated by several underlying factors rather than a unidimensional construct like fearfulness or coping style. The inclusion of physiological and neuroendocrine measures in future studies will help to elucidate the biological mechanisms underpinning individual differences in reactivity and will thereby improve our understanding and assessment of emotional states in calves.

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CHAPTER 3

Responses of calves to acute stress: individual consistency and relations between behavioral and physiological measures

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ABSTRACT

The present study examined the consistency over time of individual differences in behavioral and physiological responsiveness of calves to intuitively alarming test situations as well as the relationships between behavioral and physiological measures. Twenty Holstein Friesian heifer calves were individually subjected to the same series of two behavioral and two hypothalamo-pituitary-adrenocortical (HPA) axis reactivity tests at 3, 13 and 26 weeks of age. Novel environment (open field, OF) and novel object (NO) tests involved measurement of behavioral, plasma cortisol and heart rate responses. Plasma ACTH and/or cortisol response profiles were determined after administration of exogenous CRH and ACTH, respectively, in the HPA axis reactivity tests. Principal component analysis (PCA) was used to condense correlated measures within ages into principal components reflecting independent dimensions underlying the calves' reactivity. Cortisol responses to the OF and NO tests were positively associated with the latency to contact and negatively related to the time spent in contact with the NO. Individual differences in scores of a principal component summarising this pattern of inter-correlations, as well as differences in separate measures of adrenocortical and behavioral reactivity in the OF and NO tests proved highly consistent over time. The cardiac response to confinement in a start box prior to the OF test was positively associated with the cortisol responses to the OF and NO tests at 26 weeks of age. HPA axis reactivity to ACTH or CRH were unrelated to adrenocortical and behavioral responses to novelty. These findings strongly suggest that the responsiveness of calves was mediated by stable individual characteristics. Correlated adrenocortical and behavioral responses to novelty may reflect underlying fearfulness, defining the individual's susceptibility to the elicitation of fear. Other independent characteristics mediating reactivity may include activity or coping style (related to locomotion) and underlying sociality (associated with vocalization).

Keywords: Calves; Individual differences; Fear; Fearfulness; Coping style; Cortisol; Heart rate; HPA axis; Behavioral test; Principal component analyses

INTRODUCTION

The profound individual differences in behavioral and physiological responsiveness to challenging situations evident in all species, including humans, have been interpreted in two main ways. First, they may reflect differences in coping style, i.e., a coherent set of behavioral and physiological stress responses characteristic of a certain group of individuals and consistent over time (Koolhaas et al., 1999). Central to this concept is the notion that individuals show qualitative differences in their responses to challenge. For example, rodents may show either a passive or an active coping style. Passive coping is characterized by behavioral inhibition, (e.g., immobility, withdrawal), high parasympathetic activity and a high adrenocortical response, whereas active coping involves fight or flight, high sympathetic activity and a low adrenocortical response (Benus et al., 1991; Koolhaas et al., 1997; Koolhaas et al., 1999). The different coping styles should be regarded as alternative types of response patterns to the same challenge (Koolhaas et al., 1997; Koolhaas et al., 1999). Second, individual variation in responsiveness to challenge may reflect differences in fear and fearfulness. Fear is an internal emotional state induced by the perception of danger during exposure to a potentially threatening stimulus (Boissy, 1995; Jones, 1996). Fearfulness is the propensity to be easily frightened, i.e., to experience an emotional state of fear in a wide variety of situations (Boissy, 1995; Jones, 1996; Jones and Hocking, 1999). Debate continues over the interpretation of certain behavioral tests and measures (Archer, 1979; Gray, 1979; Jones, 1996; Ramos and Mormède, 1998) but physiological responses associated with an emotional state of fear are generally believed to include increased activity of the sympathetic nervous system, e.g., higher heart rate and catecholamine secretion, and of the hypothalamo-pituitary-adrenal (HPA) axis, e.g., elevated plasma corticosteroids (De Boer et al., 1990; Castanon and Mormède, 1994; Boissy, 1995; Boissy and Le Neindre, 1997; Ramos and Mormède, 1998; Rushen et al., 1999a). When faced with the same challenge fearful animals show more pronounced behavioral (e.g., immobility or panic) and physiological (e.g., sooner. greater, longer) responses than less fearful ones. Thus, variability in fearfulness is thought to reflect individual differences in the level of responsiveness or in response thresholds rather than in the qualitative type of response (Lyons, 1992; Boissy and Bouissou, 1995; Erhard et al., 1999).

Evidence has accumulated that coping style and underlying fearfulness represent basic constitutional traits that are to a certain extent genetically mediated (Suomi, 1991; Benus et al., 1991; Boissy, 1995; Jones, 1996; Ramos and Mormède, 1998; Jones and

Hocking, 1999) and that may affect the individual's predisposition to develop disease or stress-related pathologies (McEwen and Stellar, 1993; Koolhaas, 1994; Boissy, 1995; Kavelaars et al., 1999). Consequently, certain response features are increasingly regarded as potential selection criteria for future breeding programmes intended to improve performance and adaptability of domestic animals (Le Neindre et al., 1995; Burrow, 1997; Faure and Mills, 1998; Jones and Hocking, 1999; Wiegant and Schouten, 2000; Schutz and Pajor, 2001). However, the consequences of genetic selection for behavioral and physiological responses to challenge has received little detailed study in farm animals, with the exception of poultry (Jones, 1996; Faure and Mills, 1998; Jones and Hocking, 1999) and mink (Malmkvist and Hansen, 2001).

Progress in this area is hampered by the lack of standardised paradigms for quantifying relevant traits and by insufficient information about the relationships between behavioral or physiological characteristics and performance-related traits (Le Neindre et al., 1995; Burrow, 1997; Schutz and Pajor, 2001). On a more fundamental level, the interpretation of individual differences in behavioral and physiological responses to challenges is open to debate. Thus, the reactivity of pigs and cattle in widely employed situations, such as novel object (NO) and open field (OF) tests, has been considered indicative of coping style (Hessing et al., 1994; Hopster, 1998; Ruis et al., 2001), of underlying fearfulness (Lawrence et al., 1991; Boissy and Bouissou, 1995), or of multiple independent traits (Munksgaard and Jensen, 1996; Désautés et al., 1997; Rushen, 2000; Erhard and Schouten, 2001).

In calves exposed individually to OF and NO tests, vocalization, locomotion, and interaction with the NO represented individually stable but mutually uncorrelated behavioral characteristics (Van Reenen et al., 2004), suggesting that multiple traits underpinned the behavioral response to environmental challenge. However, the absence of physiological data weakened conclusions about underpinning biological factors. This generated two main objectives: (1) to establish the relationships between behavioral and physiological responses of calves in similar tests as those previously used, i.e., NO and OF tests, and (2) to develop a model of responsiveness to acute environmental challenge in calves, in terms of fearfulness and coping style. The physiological measures were heart rate (sympathetic nervous activity) and plasma cortisol responses (adrenocortical activation). We hypothesized that simultaneous covariation of a behavioural measure with both heart rate and cortisol responses but in opposite directions would favour an interpretation in terms of coping style (Koolhaas, 1994;

Koolhaas et al., 1997), whereas covariation in similar directions could be explained in terms of underlying fearfulness (De Boer et al., 1990; Castanon and Mormède, 1994; Boissy and Le Neindre, 1997).

We also calculated the correlations between individual HPA axis response profiles to exogenous CRH and ACTH and other measures because differences in HPA axis reactivity (or sensitivity) *per se* are thought to be linked to individual differences in OF behavior (Von Borell and Ladewig, 1992), coping style (Ruis et al., 2000), and adrenocortical activation Munksgaard and Jensen, 1996). Tests were repeatedly performed between 3 and 26 weeks to examine long-term consistency of individual differences.

MATERIALS AND METHODS

The present experiment was carried out at an experimental farm of the Animal Sciences Group of WUR in Lelystad, The Netherlands, and it was approved by the institute's Animal Care and Use Committee.

Animals, housing and management

We used 20 Holstein Friesian heifer calves born on the same experimental dairy farm of the Animal Sciences Group, in the same calving season (autumn) and with a birthweight equal to or exceeding 40 kg. Four batches of five calves each were established at a rate of one batch per successive week. Within batches, ages of individual calves differed by no more than 3 days from the group mean.

Following birth, calves were housed individually in straw-littered pens. They received colostrum for the first 2 days and were then fed according to a regular schedule for rearing calves based on provision of whole milk gradually supplemented with concentrates and ad libitum roughage (hay and maize silage). At approximately10 days, each batch of 5 calves was housed together in a straw-littered pen (3 x 3 m). The pens were adjacent and allowed visual and tactile contact between neighbouring groups. Calves were dehorned at 8 weeks and weaned at 10 weeks of age. After the calves in the fourth batch were tested at 13 weeks (see below) all 20 calves were transferred at the same time to an adjacent cubicle house.

Testing procedures

Each calf was individually subjected to the same series of two behavioral and two HPA axis reactivity tests at 3, 13 and 26 weeks of age. At each age, the tests were preceded by a 4-day habituation period to familiarize the calves with the test conditions (see below) and were completed within a period of 15 days. Within tests, calves of the same batch were tested in random order. The experimental calendar is presented in Table 3.1.

Table 3.1.

Timing of experimental procedures employed in heifer calves at each of 3, 13 and 26 weeks of age

Day[a]	Experimental procedure
-3 to 0	Habituation to testing conditions
1	Recording of baseline heart rate between 12.30 and 15.30 pm, weighing
4	Open field test between 13.00 and 15.00 pm, insertion of jugular catheter
5	ACTH challenge test or control test between 09.00 and 11.00 am
6	ACTH challenge test or control test between 09.00 and 11.00 am, removal of catheter
13	Novel object test between 13.00 and 15.00 pm, insertion of jugular catheter
14	CRH challenge test or control test between 09.00 and 11.00 am
15	CRH challenge test or control test between 09.00 and 11.00 am, removal of catheter

[a]Calves were tested at a mean age on day 4 of 3, 13 and 26 weeks, respectively

Behavioral tests

Behavioral tests were performed in a large enclosed room, approximately 30 m from the home pens. This room contained separate test arenas measuring 3 x 4.5 m and 6 x 6 m for the NO and OF tests, respectively, and with 2 m high wooden walls. A start box, with 2 m high walls and adjustable length and width, was connected to one corner of the OF and allowed access to it through remotely controlled, pneumatically-operated swing doors. The calves could not turn around inside the start box. The OF and the start box had solid concrete floors that were cleaned with a high pressure hose before each test. A manually operated slide door allowed entrance into the NO test arena which had

a straw-littered floor. Faeces and urine were removed after each NO test, a little fresh straw was added, and the bedding material was redistributed evenly. The straw was refreshed between test periods.

All behavioral tests were recorded onto videotape using overhead cameras, and a microphone recorded vocalizations. Behavioral measures were analyzed using the Observer Software System for Behavioral Research (Noldus Information Technology, Wageningen, The Netherlands). Halters were fitted to the calves at the beginning of each habituation period to facilitate handling; the calves wore these throughout each 15-day episode of testing. Calves were led to and from the test room by a rope attached to the halter.

A blood sample was taken by jugular venepuncture before and immediately after each test in the home pen and in the arena. Blood was collected in 10-ml evacuated tubes (Vacuette®; Greiner BV, Alphen a/d Rijn, The Netherlands) containing the anticoagulant EDTA (ethylenediaminetetraacetic acid). Blood samples were held on ice, and centrifuged for 12 min at 3000 rpm. Plasma was extracted and stored in aliquots at -20ºC for subsequent cortisol assay. Blood sampling was carried out by 2 persons, one restrained the calf while the other took blood. The average time between entering the home pen or test arena and completion of blood sampling was 48 s. After thorough habituation to human handling (see below) the calves were generally docile and easy to restrain during sampling. To facilitate pre-test blood sampling in the cubicle house (at 26 weeks) calves of the same batch to be tested were separated from the rest of the herd by a fence.

Open field test. Calves were individually confined in the start box for 3 min. The swing doors were then automatically opened and the calf could voluntarily enter the OF or remain in the start box. If it entered the OF the doors were closed behind it. Unlike Boissy and Bouissou (1995) and Jensen et al. (1997) we refrained from forcing the calves into the OF after a prescribed time in order to avoid potential bias due to differential effects of voluntary and forced entry. The calf was observed for 10 min after the doors were opened. Behavioral measures included: the latency to enter the OF, the accumulated times spent in locomotion and in contact (sniffing, licking) with the floor or the walls, and the numbers of vocalizations, defecations and urinations (see Table 3.2 for full definition of measures).

Novel object test. The methodology resembled that used in our previous study (Van Reenen et al., 2004) and with pigs (Hessing et al., 1994; Lawrence et al., 1994). Calves were introduced individually into the test arena and then remained undisturbed

for 3 minutes. A novel object connected to a rope was then lowered rapidly from the ceiling to the floor at the center of the arena. It consisted of a tambourine attached to a yellow plastic container (25 x 25 x 50 cm). It was weighted sufficiently to cause a slight noise when it hit the floor, thereby attracting the attention of the calf, and it was then immediately pulled back up to a height of 0.5 m and left in that position for the 10 min test. We then recorded: the latency to contact the object, the accumulated times spent in contact with the object and in locomotion, and the numbers of vocalizations, defecations and urinations (see Table 3.2).

Table 3.2

Definition of behavioral measures and events recorded in heifer calves during open field and novel object tests

Measure or event	Definition
Recorded during both tests	
Locomotion	Movement of front legs or all four legs
Vocalization	All types of vocalizations
Defecation and Urination	Defecations and Urinations during the test[a]
Open field test	
Start of 3-min pre-test phase	Moment when calf was isolated in starting box
Start of test	Moment when automatic doors to the open field were opened
Latency to enter the open field	Time from start of test until the calf placed all 4 legs in the open field
In contact with floor/walls	Sniffing or touching floor or walls with nose or tongue
Novel object test	
Start of 3-min pre-test phase	Moment when calf was isolated in test arena
Start of test	Moment when novel object had been lowered
Latency to first contact with the object	Time from start of test to the first contact with the object with nose or tongue
In contact with novel object	Sniffing, touching or rubbing the object with nose, tongue or head

[a]Including 3-min pre-test phase

HPA axis reactivity tests.

Two different HPA axis reactivity tests were performed in the home pen: cortisol responses to exogenous ACTH, and ACTH, and cortisol responses to exogenous CRH. Each calf was subjected to a stimulation (ACTH or CRH) test and a control test (vehicle only) on consecutive days. The order was balanced across calves. Calves were fitted with an indwelling jugular catheter (1.4 m, polythene; Portex Ltd, Hythe, UK) on the evening before the first day of HPA axis reactivity testing. Catheters were non-surgically inserted via a 12 gauge needle (Intraflon; Vygon, Ecouen, France), placed inside a custom-made pouch attached to the animal's neck and covered with an elastic bandage for protection. Catheters were filled with citrate solution (0.1 *M* in sterile physiological saline) to prevent blood clotting. During testing in the group pens at 3 and 13 weeks calves of the same batch were tethered side by side to the front of the pen with 0.6 m ropes attached to the halters. Removable steel bar partitions allowed visual and limited tactile contact between neighbouring calves. At 26 weeks, tethered calves were placed in adjacent cubicles during HPA axis reactivity tests. The cubicles were blocked at the back to prevent interference from other calves. After two consecutive days of HPA axis reactivity testing (Table 3.1) the catheters were removed and antibiotics were administered as a precautionary measure.

Forty-five minutes before the start of the test at 09.00, calves of the same batch were restrained in the experimental setup. Catheters were accessible for remote blood sampling. In the ACTH challenge test, calves received an intravenous injection of 0.016 IU $ACTH_{1-24}$ (Synacthen; Ciba-Geigy, Basel, Switzerland)/kg body weight$^{0.75}$ (Ladewig and Smidt, 1989) in 5 ml sterile 0.9% saline solution. In the CRH challenge test, a dose of 0.03 µg bovine CRH (Bachem AG, Bubendorf, Switzerland)/kg body weight (Veissier et al., 1999) in 5 ml saline was given intravenously. Blood samples were collected into plastic 10-ml evacuated tubes containing EDTA at 15 minutes before (-15) and at 10, 20, 30, 45, 60 and 75 min after administration of ACTH, CRH or vehicle. Blood samples were kept on ice and centrifuged within 1 h of collection for 12 min at 3000 rpm in a cooled centrifuge (4ºC). Plasma samples were stored either at -20ºC or at -80ºC until assay for cortisol (all samples) or ACTH (duplicates of samples obtained in CRH challenge tests), respectively.

Heart rate

At each age, heart rate was continuously monitored during a period of baseline recording in the home pen 3 days before the OF test (Table 3.1) and during the tests. Calves were equipped with a non-invasive heart rate monitoring system (Polar Electro Oy, Helsinki, Finland) validated for use in bovines (Hopster and Blokhuis, 1994). Mean heart rates were recorded at 5-s intervals. When baseline recordings were made at 26 weeks, calves wearing monitors were separated from the rest of the herd by a fence. Recording of baseline heart rate continued until the animal lay down for at least 15 min. The recording of heart rate during a behavioral test began in the home pen immediately following collection of the pre-test blood sample. All heart rate data were transferred to a computer for further analyses.

Habituation

A fixed daily schedule of habituation was carried out over a 4-day period prior to testing at each age (Table 3.1). Habituation involved leading individual calves in and out of the home pen twice a day with a rope attached to the halter, and attaching the heart rate belts and neck bandages for 3 hours. In addition, calves were restrained in the conditions used for challenge tests for one hour in the morning on 3 separate days. While restrained, calves generally lay down and appeared calm, e.g., they ruminated. Calves were not exposed to the test room prior to behavioral testing.

Hormone assays

Plasma levels of cortisol were determined using a time resolved fluoroimmunoassay in unextracted bovine plasma (Erkens et al., 1998). Samples were run in duplicate. The lower detection limit for a 20 µg sample was 0.5 ng/ml. Intra- and inter-assay coefficients of variation were 8.2 and 9.8% for high (71.1 ng/ml), 7.9 and 5.0% for intermediate (39.2 ng/ml), and 11.3 and 8.6% for low (10.3 ng/ml) control samples.

Concentrations of plasma ACTH were analysed using a commercially available assay for human plasma (Nichols Institute Diagnostics, San Juan Capistrano, USA) validated for bovine plasma. Mean concentrations of low, high and intermediate control samples were 11.4, 83.0 and 45.8 pg/ml, respectively, and corresponding inter-assay coefficients of variation were 11.2, 3.8, and 4.1%. The intra-assay coefficient of variation for intermediate control samples was 2.6%. The detection limit was 1 pg/ml.

Data processing and statistical analyses

The accumulated times spent in locomotion, in contact with the floor or walls, and in contact with the NO were all expressed as percentages of the total test duration. Average heart rates were calculated for a 10 min baseline period starting 2 min after the calf was seen to be lying down, for the 3 min period when it was confined in the start box, for 10 min after the swing doors were opened (OF test), and for 10 min after the NO was lowered (NO test). Heart rate responses were calculated by subtracting baseline values from average heart rates during confinement in the start box and during the OF and NO tests, respectively. Adrenocortical responses to the OF and NO tests were calculated by subtracting pre-test from post-test plasma cortisol levels. To summarize the plasma cortisol and ACTH responses to ACTH or CRH challenge tests, integrated areas under the 'hormone against time after administration of exogenous ACTH or CRH' curves were calculated. The cortisol/ACTH ratio after injection of exogenous CRH was calculated by dividing the area under the plasma cortisol against time curve by the area under the plasma ACTH against time curve. This ratio represents a measure of adrenocortical sensitivity to endogenous ACTH (Janssens et al., 1995; Veissier et al., 1999).

Calves rarely urinated or defecated more than once. Moreover, urination or defecation were absent in 49% of the behavioral tests. Therefore, frequencies of urination and defecation were transformed into a single discrete (binary) characteristic, with values "0" (no urination/defecation) or "1" (urination/defecation).

Samples obtained during control HPA axis reactivity tests (vehicle injection) were initially only analyzed for plasma cortisol. Plasma ACTH was determined in 20% of samples at each age after administration of vehicle on the day before or after the CRH reactivity test (i.e., complete response curves in 4 randomly selected calves, different animals at different ages). Differences between sampling times were calculated per animal and analysed with the Wilcoxon's matched pairs signed rank test (Conover, 1980). Average cortisol or ACTH levels at each time-point after injection of vehicle never differed significantly from those found before injection of vehicle, ACTH or CRH (results not shown), indicating that sampling *per se* did not elicit a pituitary-adrenocortical response over baseline. Therefore, pituitary-adrenocortical responses to vehicle were not considered in subsequent statistical analyses of data with regard to HPA axis reactivity tests.

Potential batch effects were examined at each test age. Latency measures were log transformed to obtain homogeneity of variances whereas the number of vocalisations (i.e., count data) were analysed as overdispersed Poisson data on a logarithmic scale. For analysis of percentages of test time (fractions) we used a logistic regression model with a multiplicative overdispersion factor, where the variance function is a multiple of the binomial variance function. Batches were entered as levels of an experimental factor in all models. Analyses of logistic and log linear models were based on maximum quasi likelihood, with overdispersion parameters estimated from Pearson's generalized chi-square statistic (McGullagh and Nelder, 1989). Latencies that reached the upper limit were introduced into the model as censored observations according to Taylor (1973). No significant differences (F-test in analyses of variance, and quasi likelihood ratio test in logistic and log linear models) were found between batches. Therefore, batch was not included as a source of variation in subsequent analyses.

Genstat (Genstat Committee, 2000) was used for all statistical calculations.

Analysis of differences between ages within animals. Measures within the same test at different ages were compared using the Wilcoxon matched-pairs signed-ranks test (Conover, 1980).

Principal component analyses. Principal component analysis (PCA) (Jolliffe, 1986) was used to condense correlated measures into their principal components. These linear combinations of the original measures reflect independent characteristics (or dimensions) underlying the correlation matrix. The loading of each measure on a principal component represents the correlation between the latent characteristic and the original measure and thus indicates the importance of a measure for a principal component. Five measures were included in a PCA: the latency to contact the NO, the time spent touching it, locomotion in the OF, and the cortisol responses to each of the OF and NO tests. A separate PCA was carried out at each age. Measures were scaled prior to PCA, i.e., the analysis was performed on the Pearson correlation matrix. Only principal components with eigenvalues equal to or larger than 1 were retained for further analyses. Prior to PCA, the latency to contact the NO object was log transformed and the percentage of time spent in contact with it was logit transformed (log(y/(1-y), replacing y = 0 by y = 0.1 * minimum of positive fractions). Latencies that reached the upper limit were introduced as censored observations (Taylor, 1973); this hardly affected the results so it was concluded that censored latencies did not compromise PCA.

Relations between measures and consistency of measures over time. To assess the relationship between heart rate and physical activity, heart rate responses to the OF and NO tests were analysed with a regression model that included as a covariate the time spent in locomotion during each test. Residuals e = y - m, where y denotes the observed heart rate response and m the corresponding estimated mean, were saved as new measures representing heart rate corrected for physical activity (i.e., nonmotor heart rate, see Baldock and Sibly, 1990; Visser et al., 2002)

Spearman rank correlations (Conover, 1980) were calculated for pairs of measures, including residual heart rates and scores of principal components, to determine the relationships between different behavioral and physiological measures and to establish whether individual differences were consistent over time.

Within each age, the relationships between urination/defecation and other measures were examined with an analysis of variance model that included urination/defecation as a fixed effect with two levels (urination/defecation or no urination/defecation). The methodology was identical to that used for the analysis of effects of batches.

RESULTS

Differences in levels of measures between ages

Tables 3.3 and 3.4 show the behavioral and physiological measures at each age. Relative to the 3-week value the latency to enter the OF was markedly lower at 13 and 26 weeks. Calves vocalized more often during OF and NO tests at 26 than at 3 weeks (Table 3.3).

Table 3.3

Behavioral responses (mean ± S.E.) of heifer calves during open field and novel object tests at 3, 13 and 26 weeks of age

	Age					
	3 Weeks		13 Weeks		26 Weeks	
Measure	Mean	S.E.	Mean	S.E.	Mean	S.E.
Open field test						
Latency to enter the open field arena (s)	268[a]	59.0	14[b]	3.0	9[b]	1.6
Locomotion (% of time)	17.4	3.51	25.5	1.88	23.7	1.90
In contact with floor/walls (% of time)	24.7	2.83	24.8	2.85	23.9	2.05
Vocalizations (number)	2.7[a]	1.23	7.1[a,b]	2.12	14.7[b]	2.62
Novel object test						
Locomotion (% of time)	15.7	2.52	12.2	1.22	12.0	1.33
In contact with novel object (% of time)	4.4[a]	1.07	1.9[b]	0.72	2.1[b]	0.96
Latency to first contact with the object (s)	105[a]	31.9	231[b]	54.8	189[a,b]	54.5
Vocalizations (number)	5.1[a]	3.64	2.1[a]	0.18	11.4[b]	2.85

(s) = seconds, (no) = number

[a,b,c]Different superscripts within a row indicate a significant difference ($P < 0.05$) between ages

Heart rate responses to the OF and NO tests decreased with age whereas heart rate responses to confinement in the start box were similar across test ages (Table 3.4). Plasma cortisol responses to the OF, NO and HPA axis reactivity tests were significantly lower at 3 than at 13 and 26 weeks (Table 3.4, Figure 3.1). Correspondingly, the lowest plasma cortisol/ACTH ratio after administration of exogenous CRH was apparent in 3-week-old calves (Table 3.4).

Table 3.4

Baseline heart rate and physiological responses (mean ± S.E.) of heifer calves during open field and novel object tests and in ACTH and CRH challenge tests, at 3, 13 and 26 weeks of age

	Age					
	3 Weeks		13 Weeks		26 Weeks	
Measure	Mean	S.E.	Mean	S.E.	Mean	S.E.
Baseline heart rate (beats/min)	88.1	4.04	91.6	2.75	91.7	2.35
Open field test						
Plasma cortisol after test[1] (ng/ml)	5.9[a]	1.02	10.7[b]	1.71	12.3[b]	2.31
Heart rate in startbox[2] (beats/min)	36.7	3.18	34.3	3.88	33.9	3.36
Heart rate during test[2] (beats/min)	28.3[a]	3.81	20.1[a,b]	3.32	14.2[b]	2.50
Novel object test						
Plasma cortisol after test[1] (ng/ml)	5.6[a]	0.94	10.2[b]	1.59	8.1[a,b]	1.24
Heart rate during test[2] (beats/min)	54.7[a]	3.37	34.0[b]	2.77	20.3[c]	3.20
ACTH challenge test						
Plasma cortisol[3]	192.8[a]	28.97	281.3[b]	21.51	221.5[a,b]	19.62
CRH challenge test						
Plasma ACTH[3]	668.4	63.96	674.4	60.26	838.5	85.33
Plasma cortisol[3]	528.4[a]	40.33	1006.0[b]	49.51	1025.0[b]	64.02
Ratio between cortisol and ACTH[4]	0.83[a]	0.049	1.67[b]	0.151	1.40[c]	0.125

[1]Elevation from corresponding pre-test plasma cortisol level

[2]Elevation from baseline heart rate recorded at the same age

[3]Area under hormone against time after administration of ACTH or CRH curve

[4]Area under plasma cortisol against time curve divided by area under plasma ACTH against time curve

[a,b,c]Different superscripts within a row indicate a significant difference ($P < 0.05$) between ages

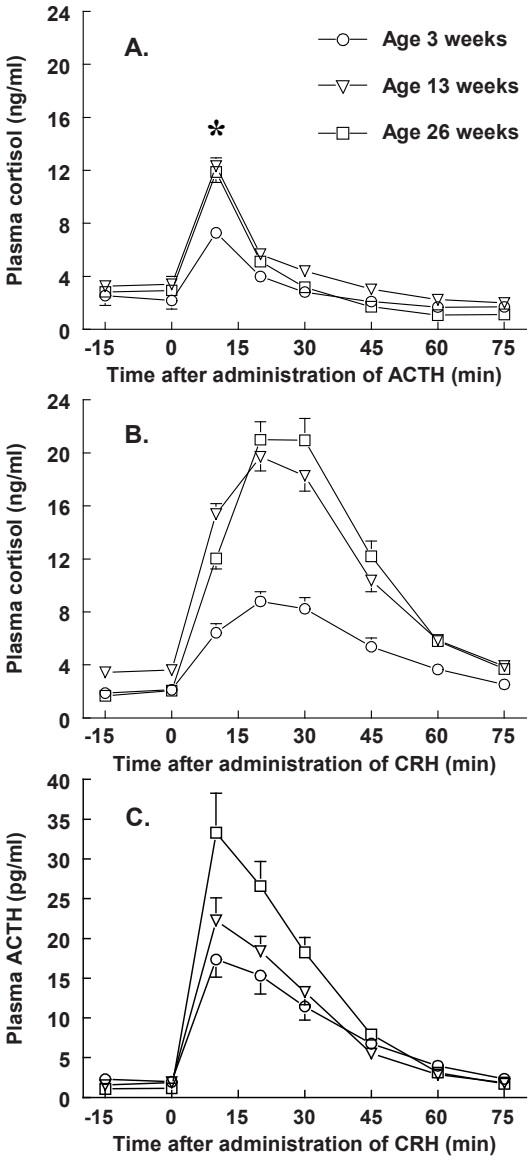

Figure 3.1 Reactivity of the hypothalamo-pituitary-adrenal axis (mean ± S.E.) to exogenous ACTH or CRH in heifer calves at the ages of 3, 13 and 26 weeks: A. plasma cortisol after intravenous administration of 0.016 I.U/kg$^{0.75}$ $ACTH_{1-24}$; B. plasma cortisol after intravenous administration of 0.03 µg/kg bovine CRH; C. plasma ACTH after intravenous administration of 0.03 µg/kg bovine CRH. *: Significant differences ($P < 0.01$) between the ages of 3 and 13 weeks, and between the ages of 3 and 26 weeks, in plasma cortisol 10 min after administration of ACTH.

Principal Component Analyses

The time spent touching the NO, the latency to contact it, and the cortisol responses to the NO and OF tests were coherently interrelated at 13 and 26 weeks (Table 3.5). Locomotion during the OF test was unrelated to the above measures. In line with this pattern, PCA produced two principal components with eigenvalues equal to or larger than 1 at each age, (2.28 and 1.13, 2.87 and 1.08, and 2.82 and 0.96 at 3, 13 and 26 weeks, respectively). Percentages of total variance explained by the first two principal components were 68, 79 and 78% at 3, 13 and 26 weeks, respectively. With the exception of the cortisol response to the OF test at 3 weeks, the loadings of measures on the first and second principal component were highly similar across ages (Figure 3.2). Locomotion during the OF test exclusively loaded on the second principal component. The first principal component had high negative loadings for the latency to contact the NO and the cortisol responses to the OF and NO, and a high positive loading for time spent touching the NO. Thus, calves with low scores on the first principal component exhibited high cortisol responses to the OF and NO tests and were reluctant to interact with the object, and vice versa.

Consistency of measures over time

Between 13 and 26 weeks of age, individual differences in 6 of 8 behavioral and in 4 of 9 physiological test measures were consistent over time (Table 3.6). Stable individual differences in heart rate response to confinement in the start box were also found between 13 and 26 weeks. On the other hand, individual differences in heart rate responses to the OF and NO (Table 3.6) and in associated residuals obtained after correction for locomotion were not consistent over any test age interval. Measures of HPA axis reactivity to exogenous ACTH or CRH correlated poorly across age with the exception of the cortisol/ACTH ratio after administration of CRH (Table 3.6). Rank orders for scores of the first principal component extracted by PCA were significantly correlated across all 3 test ages (Table 3.6), demonstrating long-term consistency of a multivariate response profile associated with behavioral and adrenocortical reactivity to novelty.

Table 3.5

Spearman rank correlations[a] between behavioral and physiological measures recorded in heifer calves during novel object test and open field tests at 3, 13 and 26 weeks of age

Measure	1	2	3	4
1. In contact with novel object (% of time)				
2. Latency to contact novel object (s)	- 0.75***			
	- 0.77***			
	- 0.87***			
3. Plasma cortisol after novel object test[b] (ng/ml)	- 0.54*	0.36		
	- 0.77***	0.58**		
	- 0.49*	0.64**		
4. Plasma cortisol after open field test[b] (ng/ml)	- 0.09	0.22	0.25	
	- 0.58*	0.30	0.80***	
	- 0.61**	0.63**	0.69**	
5. Locomotion during open field test (% of time)	- 0.07	- 0.16	- 0.07	- 0.30
	- 0.07	0.13	- 0.10	0.13
	0.11	- 0.13	- 0.06	- 0.19

[a]Within each cell of the matrix, the correlations in the first, second and third row represent the correlations between measures obtained at the ages of 3, 13 and 26 weeks, respectively

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

[b]Elevation from corresponding pre-test plasma cortisol level

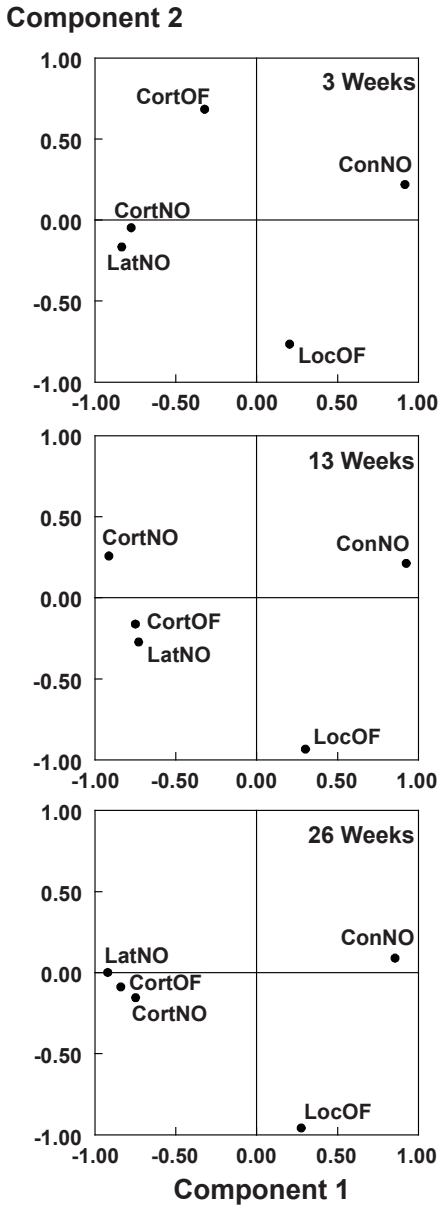

Figure 3.2 Distributions in relation to the first two principal components extracted after principal component analyses (PCA) of measures obtained in heifer calves during an open field test and a novel object test at the ages of 3, 13 and 26 weeks of age. Loadings of each measure on the first and second principal component serve as coordinates on the X-axis and Y-axis, respectively. Labels of measures: ConNO = In contact with novel object; LatNO = Latency to first contact with novel object; LocOF = Locomotion during open field test; CortNO = Cortisol response to novel object test; CortOF = Cortisol response to open field test.

Table 3.6

Consistency[a] over time of individual differences in behavioral and physiological measures recorded in heifer calves at the ages of 3, 13 and 26 weeks, and in scores of principal components

	Interval between ages		
Measures	3 - 13 weeks	3 - 26 weeks	13 - 26 weeks
Open field test			
Latency to enter the open field arena (s)	0.32	0.33	0.57**
Locomotion (% of time)	- 0.29	0.09	0.34
In contact with floor/walls (% of time)	0.05	- 0.01	0.51*
Vocalizations (number)	0.32	0.32	0.78***
Plasma cortisol after test[b] (ng/ml)	0.09	- 0.07	0.54*
Heart rate in startbox[c] (beats/min)	0.27	0.36	0.57**
Heart rate during test[c] (beats/min)	0.12	0.21	0.43
Novel object test			
Locomotion (% of time)	0.61**	0.43	0.35
In contact with the object (% of time)	0.46*	0.33	0.76***
Latency to first contact with the object (s)	0.31	0.51*	0.64**
Vocalizations (number)	0.75***	0.65**	0.71***
Plasma cortisol after test[b] (ng/ml)	0.46*	0.52*	0.74***
Heart rate during test[c] (beats/min)	0.04	0.21	- 0.42
ACTH challenge test			
Plasma cortisol[d]	- 0.05	- 0.12	- 0.24
CRH challenge test			
Plasma ACTH[d]	0.24	0.45	0.14
Plasma cortisol[d]	0.03	0.25	- 0.10
Ratio between cortisol and ACTH[e]	0.56*	0.68**	0.49*
Scores of principal components[f]			
First principal component	0.55*	0.68**	0.87***
Second principal component	0.10	0.12	0.27

[a]Spearman rank correlations across measures taken at 3, 13 and 26 weeks of age
* P <0.05; ** P <0.01; *** P <0.001
[b]Elevation from corresponding pre-test plasma cortisol level
[c]Elevation from baseline heart rate recorded at the same age
[d]Area under hormone against time after administration of ACTH or CRH curve
[e]Area under plasma cortisol against time curve divided by area under plasma ACTH against time curve
[f]Principal components were extracted by principal component analyses of five measures. See Figure 3.2 for loadings of measures on the first and second principal component. High scores of the first principal component indicate low cortisol responses to the novel object and open field tests, short latencies to first contact with the novel object, and high percentages of time spent in contact with it. High scores of the second principal component reflect low levels of locomotion during the open field test.

Relations between measures and inter-test consistency

Regression analyses revealed only one significant relationship between locomotion and heart rate: at 3 weeks locomotion in the OF accounted for 52% of total variance in heart rate response to that test; estimate of regression coefficient ± S.E.: 0.75 ± 0.12; $P < 0.001$. In 4 of the 5 remaining regression models (NO test at all ages, OF test at 26 weeks) residual variance exceeded that of the response variate, i.e., there was no indication of a relationship.

Measures of heart rate recorded during the OF and NO tests and during confinement in the start box were significantly interrelated at 3 and 13 weeks (Table 3.7). However, at 26 weeks, measures of heart rate in the NO test no longer correlated with those recorded during confinement in the start box or in the OF (Table 3.7).

There were no significant correlations within ages between measures of heart rate in OF and NO tests and those of behavioral or HPA axis reactivity. At 26, but not at 3 or 13 weeks, the heart rate response to confinement in the start box was significantly correlated with the time spent in contact with the NO and with the cortisol responses in both the OF and NO tests (rank correlations -0.52, 0.50 and 0.53, respectively, $P < 0.05$).

At 13 and 26 weeks, calves were consistent in their tendency to show high or low levels of vocalization during the OF and NO tests (rank correlations between tests of 0.69 and 0.55, respectively, $P < 0.01$). There were no significant relationships between vocalization during OF or NO tests and other behavioral or physiological measures. Inter-test consistency of locomotion was found at 26 weeks (rank correlation between tests 0.49, $P < 0.05$).

Measures of HPA axis reactivity to exogenous ACTH or CRH, including the cortisol/ACTH ratio, were uncorrelated with the cortisol responses in the OF and NO tests or with any of the cardiac or behavioral measures.

Scores of the first principal component, summarizing behavioral and adrenocortical responses to novelty, were correlated with two other measures not included in the PCA: the heart rate response to confinement in the start box at 26 weeks (rank correlation -0.55, $P < 0.05$) and locomotion during the NO test at 3 and 26 weeks (rank correlations 0.58 and 0.67, respectively, $P < 0.01$). Thus, calves showing high cortisol responses in OF and NO tests and high avoidance of the NO showed reduced locomotion during the NO test and, at 26 weeks, high heart rate responses to confinement in the start box. Scores of the second principal component, with high negative loadings for locomotion in the OF, were negatively correlated with the heart rate

response to this test at 3 weeks. This is consistent with the results of the regression analyses.

At 13 weeks, there was a positive relationship between urination/defecation and the cortisol response to the OF: calves showing no urination or defecation had lower cortisol responses than those that excreted (6.3 versus 15.1 ng/ml, SE = 1.94, $F_{1,18}$ = 10.3, $P < 0.01$). No other relationships were detected.

Table 3.7

Spearman rank correlations between measures of heart rate recorded in heifer calves during a novel object test (NOT) and an open field test (OFT) at 3, 13 and 26 weeks of age

	Age		
Measures	3 weeks	13 weeks	26 weeks
Heart rate[a] in start box correlated with heart rate during OFT	0.67**	0.82***	0.53*
Heart rate in start box correlated with heart rate during NOT	0.78***	0.52*	0.00
Heart rate during OFT correlated with heart rate during NOT	0.63**	0.55*	- 0.03
Heart rate in start box correlated with residual heart rate[b] OFT	0.69**	0.87***	0.56*
Heart rate in start box correlated with residual heart rate NOT	0.76***	0.50*	- 0.07
Residual heart rate OFT correlated with residual heart rate NOT	0.33	0.54*	- 0.09

[a]Heart rates are elevations from baseline

[b]Residual heart rates were saved after regression analyses using heart rate responses to OFT and NOT as dependent variables and time spent in locomotion during OFT and NOT as explanatory variables, respectively

* P <0.05; ** P <0.01; *** P <0.001

DISCUSSION

The present results demonstrate a clear relationship between calves' cortisol responses to the OF and NO tests and their behavioral interaction with the NO. High cortisol responses were associated with long latencies to contact and short times spent touching the NO, and vice versa. With the use of PCA, this pattern of inter-correlations could be adequately summarized into an aggregate measure (i.e., the first principal component) of adrenocortical and behavioral reactivity to novelty. Importantly, differences in scores of this aggregate measure were highly consistent across age. These findings strongly suggest that the adrenocortical and behavioral responses of calves to novelty were mediated by a common and individually stable underlying biological process or characteristic.

Avoidance of novel objects and enhanced adrenocortical reactivity to challenge are widely regarded as behavioral and physiological correlates, respectively, of increased fearfulness in mammals and birds (Lyons et al., 1988; De Boer et al., 1990; Castanon and Mormède, 1994; Boissy, 1995; Boissy and Bouissou, 1995; Jones, 1996; Rushen et al., 1999a). Recent studies of the relationship between behavioral style or personality and cortisol reactivity support this notion and, like the present findings, indicate long-term consistency of individual differences in underlying characteristics. For example, the cortisol response of 5-month-old gilts to a novel environment was negatively associated with a profile of maternal behaviors labelled "calmness" recorded in the same animals approximately 19 months later (Spinka et al., 2000). Furthermore, the cortisol responses of 6 and 12-month old capuchin monkeys to brief social isolations were positively correlated with scores of a subjectively rated personality trait labelled "fearful" in the same individuals over a 5-year period (Byrne and Suomi, 2002). Thus, we consider it likely that calves that took a long time to contact the NO, spent only a short time touching it and showed high cortisol responses in the OF and NO tests were more fearful than those exhibiting the opposite response profile. Correspondingly, fearfulness is thought to be an important intervening variable underlying differential behavioral and adrenocortical reactivity to novelty.

Alternatively, it has been proposed that pigs with long and short latencies to contact a novel stimulus (object or human) represent individuals with a passive or active coping style, respectively (Hessing et al., 1994; Ruis et al., 2000). The increased cortisol (Ruis et al., 2000) but decreased heart rate responses (Hessing et al., 1994) found in pigs with long latencies to contact the stimulus supported an interpretation in terms of coping style (Koolhaas et al., 1997, 1999). Conversely, the present findings do not

support a similar interpretation of individual differences in behavioral and physiological responses of calves. Although calves showing long latencies to contact and little subsequent interaction with the NO had higher adrenocortical responses, they either showed similar or higher heart rate responses to confinement in the start box (at least at 26 weeks). This finding is consistent with the positive relationship found in adult cows between cardiac reactivity to confinement in a start box and the cortisol response to an ensuing OF test (Hopster, 1998). Therefore, we argue that the reactions of our calves to the NO reflected quantitative rather than qualitative differences in the type of response. In line with our previous argument, it is likely that correlated levels of physiological reactivity and avoidance of the NO were a function of underlying fearfulness, with fearful calves adhering to the same strategy and mechanism of response.

Our findings closely parallel those on individual differences in temperament (response to novelty) in human infants and primates (Clarke and Boinski, 1995; Rothbart et al., 2000). Indeed, consistent response styles have been identified in children and rhesus monkeys, and characterized using terms such as “inhibited” or “fearful” versus “uninhibited” or “relaxed” (Kagan et al., 1988, 1998; Suomi, 1991). Thus, inhibited or fearful individuals were reluctant to approach and interact with novel objects, displayed behavioral signs of distress more frequently, and had heightened cortisol and heart rate responses.

The time spent in locomotion during the NO test was correlated here with the aggregate measure of behavioral and adrenocortical responsiveness to novelty derived from PCA; calves showing high cortisol responses and high avoidance of the NO showed low locomotion, and vice versa. Assuming that our aggregate measure reflected underlying fearfulness, this agrees with the suggestion that reduced locomotor activity in heifers indicates high fearfulness (Boissy and Bouissou, 1995). Interestingly, risk assessment and fear-related behavior of rats and mice in an elevated plus-maze was strongly positively correlated with their corticosterone response to the maze (Blanchard et al., 1991), probably reflecting the role of corticosteroids in facilitating information-processing in potentially dangerous environments (Rodgers et al., 1999; Korte, 2001). Long contact latencies and low locomotor activity in our calves may also have been related to elevated risk assessment and vigilance. This suggestion is supported by our subjective impression that the calves seemed highly alert during NO testing, often fixating on the NO before interacting with it.

Unlike the NO test, locomotion in the OF was unrelated to cortisol responses or to measures of behavioral reactivity towards the NO. This agrees with studies in cows

(Hopster, 1998), rats (Stern et al., 1973) and monkeys (Byrne and Suomi, 1999) where locomotion in an OF or during brief social isolation was dissociated from adrenocortical responses. It also supports our previous observation (Van Reenen et al., 2004) of dissociation between calves' locomotion in an OF and their interaction with a NO. However, unlike our previous study (Van Reenen et al., 2004) individual differences in OF locomotion were not consistent over time in the present study. This apparent inconsistency may simply reflect methodological differences; calves could enter the OF voluntarily here whereas they were encouraged to enter the arena in our previous experiment (Van Reenen et al., 2004). Thus, in the present study competing motivations to walk in the OF or to remain in the start box may have obscured intra-individual consistency across time. Nevertheless, subsequent longitudinal research with the same animals (in preparation) revealed that individual differences in OF locomotion at 26 weeks reliably predicted those at 29 months. Collectively, our findings support the suggestion that individual differences in OF locomotion reflected the existence of an individually stable characteristic, at least from 26 weeks onwards.

The lack of association between OF locomotion and the inter-correlated adrenocortical and behavioral responses to novelty might then be explained by assuming that the former measure was mediated independently of underlying fearfulness, for example by a "general activity" trait (Andersen et al., 2000b). Alternatively, variations in locomotion may have reflected individual differences in the qualitative type of behavioral pattern adopted in response to the perception of a fear-eliciting situation (Archer, 1979; Boissy, 1995; Ramos and Mormède, 1998). Putatively fearful calves, i.e., those with high cortisol responses, could exhibit either high or low levels of locomotor behavior; these contrasting responses might reflect fear-induced activity (e.g. attempting to escape) or fear-induced immobility, respectively. This explanation also implies that calves experiencing similar levels of fear may have used different behavioral strategies, involving different degrees of activity, to cope with the same challenge. From a behavioral point of view, this agrees with the concept of coping style (Erhard et al., 1999; Ruis et al., 2001). A possible link between OF locomotion and coping style is also supported by our previous finding (Van Reenen et al., 2004) that locomotion was positively correlated with the time spent struggling during a restraint test; the latter type of behavior has been widely implicated in the distinction between different coping styles in pigs (Hessing et al., 1994; Erhard et al., 1999; Ruis et al., 2000; Bolhuis et al., 2002).

The above argument is summarized in Figure 3.3 which provides a model of responsiveness in calves along two independent (orthogonal) axes: i) fearfulness,

defining the animal's susceptibility to the elicitation of fear, and ii) activity, defining its propensity to perform active behaviors. The response profiles of individual calves can be arranged in this two-dimensional plane according to locomotion during the OF test (activity) on the one hand and to the correlated adrenocortical and behavioral responses to novelty (fearfulness) on the other. We propose that the adoption of an active or passive coping strategy results from the interaction between fearfulness and other underlying characteristics mediating activity and that this mechanism is particularly apparent in fearful calves, i.e., those that perceive the test situation as a threat. Among less or non-fearful calves, characteristics other than those presumed to mediate coping style may also regulate OF locomotion, for instance a tendency to explore (Jensen et al., 1997). The notion that the elicitation of fear might be a prerequisite for the expression of individual differences in coping style is supported by studies of two genetically selected lines of mice showing either an active or a passive coping style (Benus et al., 1991). These lines only differed in the expression of defensive burying (a behavioral index of coping style) when tested in a stressful environment (Sluyter et al., 1996).

The numbers of vocalizations during the OF and NO tests were inter-correlated at 13 and 26 weeks of age and individually consistent over time. However, this measure was unrelated to any of the other behavioral or physiological ones. This finding concurs with our previous observation (Van Reenen et al., 2004) that consistent differences in vocalization in calves were dissociated from those in locomotion, interaction with a NO, and struggling during restraint. Therefore, in addition to fearfulness and activity, a third independent characteristic, associated with vocalization, might underpin individual differences in reactivity of calves to tests involving social separation. Sociality (i.e., the motivation to be near companions) might be a likely candidate trait, since vocalization during social isolation is generally thought to represent an important behavioral marker of this trait in bovines, sheep and poultry (Boissy and Bouissou, 1995; Erhard and Schouten, 2001; Jones and Mills, 1999; Watts and Stookey, 2000). In terms of our model of responsiveness (Figure 3.3), this would add a third dimension to the picture, orthogonal to the other two.

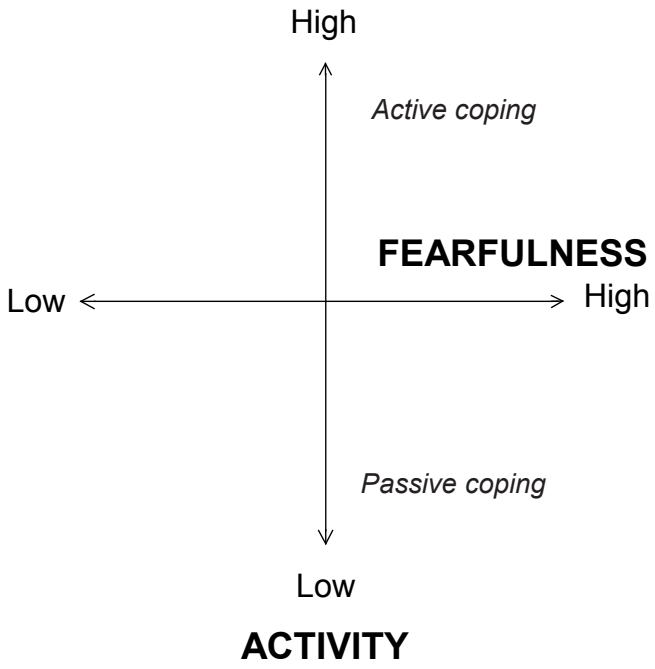

Figure 3.3 Model of responsiveness of calves along two independent (orthogonal) underlying dimensions: i) fearfulness (horizontal axis), defining the susceptibility of the individual to the elicitation fo fear, and ii) activity (vertical axis), defining the propensity of the individual to perform active behaviors or to actively resist a challenge. Response profiles of the present calves can be arranged in this two-dimensional plane according to the amount of locomotion during the open field test (activity), and levels of correlated adrenocortical and behavioral responses to novelty (fearfulness). Adoption of an active or passive coping style may result from the interaction between fearfulness and underlying characteristics mediating activity; this may be most apparent in calves with high levels of fear (right side of the two-dimensional figure). See text for further explanation.

Unlike pigs (Von Borell and Ladewig, 1992; Ruis et al., 2000), measures of HPA axis reactivity to exogenous ACTH and CRH in our calves were not correlated with cortisol or behavioral responses to the OF or NO tests. This finding further supports the notion that individual differences in the adrenocortical reactivity of calves were mediated at a high level of the central nervous system following the perception and appraisal of potential threat. Similarly, divergent selection of quail or mink for low or high fear was

associated with reduced or exaggerated adrenocortical responsiveness to (emotional) stressors without affecting adrenal sensitivity to exogenous ACTH or CRH (Launay et al., 1993; Malmkvist and Hansen, 2001). Interestingly, changes with age in the average cortisol responses of our calves to OF and NO exposure coincided with those in the average adrenocortical output after ACTH and the average cortisol/ACTH ratio after CRH challenge. Thus, age-related sensitivity of the adrenals might be an important determinant of the overall intensity of the adrenocortical response to novel or alarming situations. To the best of our knowledge, our data are the first to demonstrate this phenomenon in calves.

Assuming that our interpretations of the present findings are correct, we were unable to confirm previously presumed relationships between fearfulness and a number of commonly used behavioral and physiological measures of this characteristic. First, although elimination has been proposed as a reliable indicator of individual differences in fear among calves De Passillé et al., 1995; Rushen, 2000), urination and defecation correlated poorly with other putative measures of fearfulness in the present study. Similarly, elimination is thought to be a poor measure of fear in mice and poultry (Gray, 1979; Jones, 1996). Second, the latency to emerge into an exposed arena has been used as a measure of fear in rodents (Archer, 1973) and poultry (Jones, 1987b) but the latency to enter the OF was dissociated from other test measures here. Third, with the exception of the cardiac response to confinement in the start box, none of the heart rate measures were associated with other behavioral or physiological ones. This included nonmotor heart rate, i.e., heart rate corrected for physical activity, which is widely believed to represent an index of fear or emotional distress in a variety of species (Baldock and Sibly, 1990; Boissy and Le Neindre, 1997; Jensen et al., 1997; Visser et al., 2002). Moreover, individual differences in (nonmotor) heart rate responses to the OF and NO tests were not consistent over time. Thus, individual differences in the heart rate responses of calves appeared to be largely age or context specific. It is conceivable that the above measures were subject to developmental changes that influenced the stability of individual differences (Hinde and Bateson, 1984; McCall, 1986). Similar to our previous study (Van Reenen et al., 2004), calves exhibited a number of behavioural changes over time that may reflect a maturational effect on the strategy young bovines adopt in threatening situations, including increases in the rate of vocalization during OF and NO tests, and in the tendency to initiate locomotion in a novel environment (i.e., to leave the start box and enter the test arena). Our findings may also point to the existence of additional dimensions or traits of the emotional response in calves (Ramos and

Mormède, 1998). However, the key proposition of the present paper, i.e., that correlated adrenocortical and behavioral responses to novelty in calves reflected underlying fearfulness, is strongly supported by recent findings that treatment of calves with benzodiazepines reduced cortisol responses to isolation and novelty, and increased locomotion and the time spent interacting with the stimulus in a NO test (in preparation).

CONCLUSION

The present findings indicate that the behavioral and physiological responses of calves to potentially alarming test situations were mediated by individually stable underlying characteristics. The interrelated adrenocortical and behavioral responses to novelty are thought likely to have reflected underlying fearfulness, with calves showing high cortisol and avoidance responses in OF and NO tests being more fearful than those showing the opposite response profile. Locomotion and vocalization in the OF constituted additional, independent behavioral dimensions of reactivity, possibly reflecting activity (coping style) and sociality, respectively. Measures of fearfulness and of other underlying characteristics might be exploited to predict the animals' ability to adapt to environmental challenges and to identify selection criteria for welfare-friendly breeding programmes.

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CHAPTER 4

The benzodiazepine brotizolam reduces fear in calves exposed to a novel object test

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ABSTRACT

The present study examined the effects of the intravenous administration of the anxiolytic drug brotizolam on the behavioral and physiological responsiveness of calves to novelty in a dose response fashion. Holstein Friesian heifer calves (39-41 weeks of age; body weight 200-300 kg) received an intravenous injection of either a vehicle control (12 calves) or one of four doses of brotizolam (8 calves per dose): 0.0125, 0.05, 0.2 and 0.8 mg/100 kg body weight. They were then individually subjected to a 'combined' test involving exposure to a novel environment (open field, OF) for 5 minutes followed by the sudden introduction of a novel object (NO) that remained in place for a further 10 min. Behavioral, heart rate and plasma cortisol responses were recorded in all animals. Compared to vehicle treatment, the highest dose of brotizolam dose-dependently and significantly increased the time spent in locomotion and the distance travelled near the NO, as well as the time spent in contact with the NO. In addition, post-test plasma cortisol concentrations changed in a dose-dependent manner over time: they decreased between 0 and 10 min after the test in calves that had received the two highest doses of brotizolam, whereas they increased in vehicle-treated and low-dosage calves. There were no effects of brotizolam on vocalization or locomotion during the OF phase of the test or on vocalization following introduction of the NO. These findings strongly support the notion that interaction with a novel object in a novel arena represents a behavioral index of fear and fearfulness in calves, and that vocalization and locomotion in an OF reflect other independent characteristics.

Keywords: Calves; Fear; Anxiolytic, Benzodiazepines, Novelty, Behavior, Heart rate, Cortisol

INTRODUCTION

Fear and anxiety are internal emotional states induced by the perception of danger during exposure to an actually (fear state) or potentially (anxiety state) threatening situation, while fearfulness refers to an underlying predisposition to be easily frightened (Boissy, 1995; Jones, 1987c, 1996). Fear is highly functional and adaptive in wild animals. However, the environmental constraints imposed upon many farm animals mean that increased fearfulness and fear responses can elicit harmful side-effects including for example anxiety disorders, increased risk of injury, decreased immunocompetence, and reduced production (Boissy, 1995; Jones, 1996; Rushen et al., 1999a; Korte, 2001; Korte et al., 2005). Such problems may become particularly apparent when animals are exposed to intense or prolonged fear-eliciting stimuli in an environment that precludes the adoption of adaptive behaviors, such as escape or shelter. Thus, in short, fear and fearfulness are important determinants of the welfare of domestic animals.

A range of paradigms has been developed to measure behavioral fear and anxiety responses in laboratory rodents (Archer, 1973; Pellow et al., 1985; Sánchez, 1995). Experimental validation of such paradigms is usually thought to require a minimum of three steps (File, 1992): (1) behavioral validation involves demonstrating relationships between exposure to specific test stimuli and the induction of supposedly fear-related behaviours, as well as statistically identifying patterns of intercorrelations between multiple behavioral measures, including putatively fear-related ones, and then interpreting them in terms of underlying fear or anxiety (e.g., Archer, 1973; Pellow et al., 1985; Rodgers and Johnson, 1995; Weiss et al., 1998), (2) physiological validation, showing that animals subjected to the test concomitantly exhibit physiological signs of fear that are correlated with the behavioral measures (e.g., Pellow et al., 1985; Hennessy and Levine, 1979; File et al., 1994; Rodgers et al., 1999), and (3) pharmacological validation, revealing sensitivity of allegedly fear-related behaviors to treatment with anxiogenic and anxiolytic drugs (e.g., Pellow et al., 1985; Sánchez, 1995; Rex et al., 1996; Hendrie et al., 1997; Choleris et al., 2001; Merali et al., 2003).

In order to measure fear responses in domestic animals, tests originally developed for laboratory animals, in particular those involving brief exposure to a novel environment (open field, OF) and other novel stimuli, have been extended to cattle (Kilgour, 1975; Boissy and Bouissou, 1995; Munksgaard and Jensen, 1996; Hopster, 1998; Kilgour et al., 2006), sheep (Torres-Hernandez amd Hohenboken, 1979; Romeyer

and Bouissou, 1992), pigs (Fraser, 1974; Lawrence et al., 1991; Jensen et al., 1995a; Ruis et al., 2001). However, attempts to validate these tests in domestic animal species predominantly focussed on behavioral validation via the use of various multivariate and correlational analyses (e.g., Kilgour et al., 2006; Romeyer and Bouissou, 1992; Jones, 1987a; Jones et al., 1991; De Passillé et al., 1995; Vandenheede et al., 1998; Andersen et al., 2000b; Janczak et al., 2002; Viérin and Bouissou, 2003; Müller and Schrader, 2005). Although the incorporation of physiological measures into this effort was addressed in some studies (Moberg et al., 1980; Minton, 1994; Jones, 1996; Boissy and Le Neindre, 1997; Hopster, 1998; Müller and Schrader, 2005), it received less attention in general, while pharmacological methods have hardly been applied at all, with the exception of some work in pigs (Andersen et al., 2000a), poultry (Lehr, 1989; Marin et al., 1997) and dairy cows (Sandem et al., 2006). Consequently, controversy remains over the interpretation of putative behavioral measures of fear in most domestic animals, including cattle (Boissy and Bouissou, 1995; Munksgaard and Jensen, 1995; Hopster, 1998; Müller and Schrader, 2005; Jensen et al., 1999; Rushen, 2000).

Recently, we showed that locomotion, vocalization and behavioral interaction with a novel object (NO) exhibited by calves during independent OF and NO tests were largely uncorrelated, suggesting the involvement of multiple underlying characteristics in the animals' behavioral responses (Van Reenen et al., 2004). Subsequently, we demonstrated that the plasma cortisol responses of calves to OF and NO tests were related to the amount of interaction with the NO, but not to vocalization or locomotion in the OF (Van Reenen et al., 2005). We, therefore, hypothesized that the correlated adrenocortical and behavioral responses of calves to novelty reflected underlying fearfulness, with high cortisol responses and high avoidance of the NO corresponding to high levels of fear and vice versa (Van Reenen et al., 2005). A logical extension to our hypothesis is that, when a NO is suddenly introduced into the OF, prior treatment with an anxiolytic drug should increase the calves' interaction with it and reduce their adrenocortical responses to the overall test, without affecting vocalization or locomotion in the OF phase.

Thus, in the present experiment calves were treated with the anxiolytic drug brotizolam prior to behavioral testing. Brotizolam is a relatively novel and sensitive benzodiazepine derivative (2-bromo-4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine), with effective doses reportedly lower than those of the classical agent diazepam, and with proven clinical efficacy in a number of species,

including bovines (Böke-Kuhn et al., 1986; Danneberg et al., 1986; Schreiber and Schultz, 1989; Moloney et al., 1990; Hirouchi et al., 1992).

MATERIALS AND METHODS

The present experiment was carried out at an experimental farm of the Animal Sciences Group of WUR in Lelystad, The Netherlands, after approval by the Institute's Animal Care and Use Committee.

Animals

A total of 44 Holstein Friesian heifer calves from the same winter calf crop, and born on the same experimental dairy farm, were used. At the time of behavioral testing (see below) all calves were between 39 and 41 weeks of age and weighed between 200 and 300 kg. Calves were distributed according to age and sire across four batches of 11, 8, 13 and 12 animals, respectively; these batches were established at a regular rate of one every two weeks. Eleven days prior to testing, calves were weighed and transported batch-wise from a cubicle house at the rearing unit to another cubicle house adjacent to the testing room (5 min drive). After completion of testing calves were returned to the rearing unit. Calves were fed ad libitum with a mixture of grass silage, maize silage and some concentrates.

Treatments and testing procedures

Within batches, calves were randomly assigned to either one of four dosage groups of Mederantil® (0.2 mg brotizolam/ml propylene glycol; Boehringer Ingelheim, Ingelheim, Germany), with 8 calves per dose, or to a vehicle treatment group (propylene glycol only, 12 calves). The doses of brotizolam used were 0.0125, 0.05, 0.2 and 0.8 mg/100 kg body weight. All doses of brotizolam were diluted in 10 ml of propylene glycol. We used a range of doses because the effectiveness of benzodiazepines may differ between species, and the precise anxiolytic dose is unkown in bovines (Danneberg et al., 1986). The dose of 0.2 mg brotizolam/100 kg body weight was chosen because its administration to cattle weighing about 350 kg stimulated feed intake with no sedative side effects (Moloney et al., 1990). There were 1-2 calves per treatment group in the second batch, and 2-3 in the other batches.

To test the effect of brotizolam on responsiveness to novelty, calves were individually subjected to a combined OF and NO test (see below) following intravenous

injection with brotizolam or vehicle. Behavioral test procedures were adapted from previously used ones (Hopster, 1998; Van Reenen et al., 2004, 2005). An overview of the timing and definitions of successive experimental procedures during the behavioral test is presented in Table 4.1. All calves were tested between 12.30 and 16.30 h. Within each batch, calves were tested on three consecutive days, 2-5 animals per day. To allow for an efficient estimation of treatment effects (see below), given the inevitably unbalanced experimental design, treatments were evenly rather than randomly distributed across test days according to a cyclic design with one vehicle-treated calf on each test day. Within test days, calves were tested in a random order.

The tests were executed in a large enclosed room, approximately 30 m from the home pen, which contained a 6 x 6 m OF arena with a start box connected to one corner. The start box allowed entrance into the arena through pneumatically operated swing doors. Both the arena and the start box had 2 m high wooden walls and solid concrete floors that were cleaned with a high-pressure hose before each test.

Blood samples were taken by jugular venepuncture on 3 occasions: in the home pen before each test, immediately after the test, and 10 min after the calf had been returned to the home environment (Table 4.1). During sampling, calves were briefly restrained while standing at the self-locking feeding gate. Blood was collected in 10-ml evacuated tubes (Vacuette, Greiner BV, Alphen a/d Rijn The Netherlands) containing the anticoagulant EDTA (ethylenediaminetetraacetic acid). Blood samples were held on ice, then centrifuged for 12 min at 3000 rpm. Plasma was extracted and stored in aliquots at -20 ºC for subsequent cortisol assay.

The appropriate dose of brotizolam or vehicle was carefully administered in the home pen, after collection of the pre-test blood sample (Table 4.1). Next, the calf was loosely tied in a cubicle for a pre-test phase of 15 min, to allow onset of the anxiolytic effect of brotizolam (Böke-Kuhn et al., 1986; Danneberg et al., 1986), and subsequently led by rope and halter to the start box where it was confined for 1 min. The OF phase of the test started when the swing doors were opened. As soon as the calf had voluntarily entered the OF, the doors were closed. Five min after opening of the swing doors, a NO consisting of a blue plastic container (25 x 25 x 50 cm) connected to a rope was lowered from the ceiling to the floor at the center of the arena. When it hit the floor, it was then immediately pulled back up to a height of 1 m and left in that position for the 10 min NO test. All behavioral responses were recorded onto videotape using an overhead color camera, and a.microphone was used to record vocalizations. Behavioral measures were analysed using the EthoVision video tracking system (Noldus et al., 2001) in combination

with the Observational Software System for Behavioral Research (Noldus Information Technology, Wageningen, The Netherlands). Calves were marked prior to the test by attaching a brightly yellow-colored piece of cloth to the heart rate belt (see below) at a spot between the shoulder blades. This piece of cloth served as the visual cue for video tracking. For the analysis with EthoVision, in addition to the test arena, a circular zone was defined with the point where the NO landed as the midpoint and a 1.5 m radius. The following measures were recorded: (1) the latency to enter the OF, (2) the numbers of vocalizations, the accumulated times spent in locomotion, the total distances travelled (m) and the average speeds of locomotion (m/s) during the OF and NO phases, (3) the latency to make contact and the accumulated time spent in contact with the NO, and (4) the accumulated time spent in locomotion and the total distance travelled (m) within a 1.5 m radius from the NO (i.e., within the circular zone). Any evidence of ataxia (i.e., impaired locomotor coordination), which may reflect side effects of benzodiazepine treatment, was also recorded. Definitions of each behavioral measure are given in Table 4.2. Although our earlier OF tests (Hopster, 1998; Van Reenen et al., 2004, 2005) lasted 10 min we used 5 min here because the number of vocalizations and the time spent in locomotion during the first 5 min of the OF test had consistently proved highly significant predictors of the same measures during the entire 10 min test (unpublished findings). The present 5 min OF test resembled the 3 min phase between the introduction of the animal in the NO test arena and the moment when the NO was lowered in our previous studies (Van Reenen et al., 2004, 2005).

Heart rate was continuously monitored throughout the OF and NO phases, as well as during a period of baseline recording in the home pen 1 week before testing. We used a non-invasive heart rate monitoring system (Polar Electro Oy, Helsinki, Finland) validated for use in bovines (Hopster and Blokhuis, 1994). Mean heart rates were recorded at 5-s intervals. All heart rate data were transferred to a computer for further analysis.

All calves were fitted with a halter to facilitate handling. They were systematically habituated to the testing conditions over a 3-day period during the week before testing; this involved locking calves group-wise in the feeding gate (1h/day) with intermittent exposure to human handling, tethering of individual calves in a cubicle (20 min/day), leading individual calves in and out of the home pen with a rope attached to the halter (twice a day), and attaching the heart rate belt (2 h/day). Baseline heart rate recordings were obtained on the third day of habituation. Calves were not exposed to the test room prior to behavioral testing.

Table 4.1

Timing[a] and definition of successive experimental procedures and periods during the behavioral test employed to examine effects of brotizolam on responsiveness to acute stress in calves

Procedure or period	Starting time (min)	Definition or description
Fetching	- 21	The experimental calf is fetched and locked in the feeding gate
Pre-test blood sampling and treatment	- 20	A blood sample is collected and brotizolam or vehicle is administered intravenously
Start heart rate recording	- 18	The calf is fitted with a heart rate monitoring system, and the recording of heart rate is begun
Pre-test phase	- 17	The calf is placed in a cubicle, and loosely tethered
Transport	- 2	The calf is taken from the home pen to the start box
Confinement in start box	- 1	The calf is confined in the start box for a period of 1 min
Start of open field test	0	Moment when the swing doors giving access to the test arena are automatically opened
Start of novel object test	5	Moment when the novel object is lowered
End of novel object test, and return	15	The calf is taken back to the home pen and locked in the feeding gate
First post-test blood sampling	16	A blood sample is collected
Second post-test blood sampling	26	A blood sample is collected
End of test	27	Recording of heart rate is stopped, the heart rate monitor is removed, and the calf is released

[a] Relative to the start of the open field test.

Hormone assay

Plasma levels of cortisol were determined using a time resolved fluoro immunoassay in unextracted bovine plasma (Erkens et al., 1998). Samples were run in duplicate. The lower detection limit for a 20 µg sample was 0.5 ng/ml. Intra- and inter-assay coefficients of variation were 8.2 and 6.4% for high (71.1 ng/ml), 7.9 and 6.1% for intermediate (39.2 ng/ml), and 11.3 and 20.8% for low (10.3 ng/ml) control samples.

Data processing and statistical analyses

The accumulated times spent in locomotion and in contact with the novel object were expressed as percentages of test duration (for both OF or NO test phases, respectively). Average heart rates were calculated during: a) a 5 min baseline period obtained 1 week prior to testing, b) the 1 min period when the calf was confined in the start box, c) the 5 min period after the automatic swing doors were opened (OF phase), and d) the 10 min period following the lowering of the novel object (NO phase). Heart rate responses were calculated by subtracting baseline values from average heart rates during confinement in the start box, and during the OF and NO test phases. Cortisol responses were calculated by subtracting pre-test concentrations from each of the two post-test plasma cortisol levels obtained immediately after and at 10 min after the test, respectively. Post-test changes in plasma cortisol level were calculated by subtracting the levels immediately after the test from those found 10 min later.

To obtain homogeneity of variance, the latency to enter the OF was log transformed. A large percentage of the calves (27%) reached the upper limit of 600 sec for the latency to first contact with the novel object. Therefore, the latter measure was transformed into a binary variable with values “0” or “1” for latencies below or above the median latency, respectively. More than half of the calves did not vocalize at all during the OF and NO test phases. Therefore, the numbers of vocalizations during these phases were also expressed as a binary variable: “0” for no vocalizations and “1” for 1 or more vocalizations.

All continuous observations were analyzed using an analysis of variance model with factors for batches, test days within batches and levels of brotizolam. Heart rate responses to the OF and NO test phases were also analysed with the same model, but with the time spent in locomotion or the average speed of locomotion (during the OF or NO test phase) included as a covariate. Analysis with the latter models provided estimates of the effects of brotizolam on heart rate corrected for the level of locomotion (Baldock and

Sibly, 1990; Visser et al., 2002). Binary variables and percentages of test time were analysed with a logistic regression model. For percentages, the model comprised a multiplicative dispersion factor with respect to the binomial variance function.

Significance tests for continuous variables were based on the common F test. Inference for binary data and percentages was based on maximum quasi-likelihood. Dispersion parameters were estimated from Pearson's generalized chi-square statistic (McCullagh and Nelder, 1989). For binary data and percentages, an approximate F-test based on the quasi-likelihood ratio test was employed. Pair-wise comparisons between treatments were made using Fisher's LSD method (applied on the logistic scale for binary variables and percentages)

All statistical calculations were performed using the statistical programming language GenStat (Genstat Committee, 2000).

RESULTS

None of the calves showed any signs of ataxia during either the OF or NO phases of the test. Brotizolam treatment significantly influenced the post-test change in plasma cortisol ($P<0.001$), the heart rate response to the NO corrected for the time spent in locomotion (nonmotor heart rate, $P<0.05$), the time spent in locomotion and the total distance travelled during the NO phase ($P<0.01$ and $P<0.05$, respectively), as well as the time spent in contact with the NO ($P<0.01$), and the time spent in locomotion and the total distance travelled within a 1.5 m radius from the NO ($P<0.01$). Averages for each treatment, and the results of pair-wise comparisons between treatment groups are presented in Figures 4.1 and 4.2. In comparison with vehicle treatment, the highest dose of brotizolam (0.8 mg/100 kg body weight) increased the time spent in locomotion and the total distance travelled during the NO phase of the test (Figure 4.1), but did not significantly affect heart rate responses to the NO (Figure 4.1). The effect of brotizolam on locomotor activity during the NO phase of the test, however, did not appear to be clearly dose-dependent (Figure 4.1). Post-test plasma cortisol concentrations changed in a dose-dependent manner over time: in the two highest dose groups cortisol levels fell between 0 and 10 min after termination of the test, whereas they increased in vehicle-treated calves (Figure 4.2).

Similarly, relative to the other treatments, the highest dose of brotizolam significantly and dose-dependently increased the time spent in contact with the NO, as well as the the time spent in locomotion and the distance travelled within a 1.5 m radius from the NO (Figure 4.2).

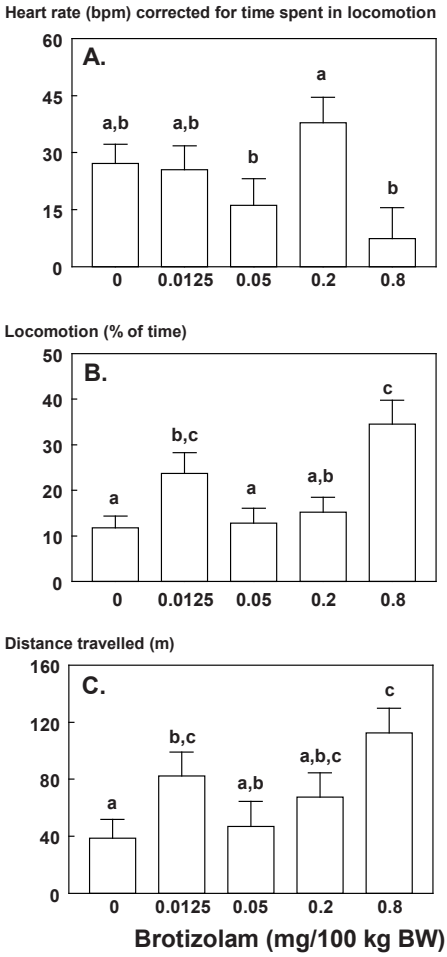

Figure 4.1 Effects of brotizolam (0.0125, 0.05, 0.2 or 0.8 mg/100 kg body weight) or vehicle (0) on behavioral and heart rate measures recorded in calves during the novel object (NO) phase of the test: A. average heart rate during the NO phase (response over baseline) corrected for the time spent in locomotion (s); B. time spent in locomotion (% of test time); C. distance travelled (m). Data are predicted means ± S.E. ^{a,b,c}. Values without a common superscript differ significantly (P<0.05, Fisher's LSD).

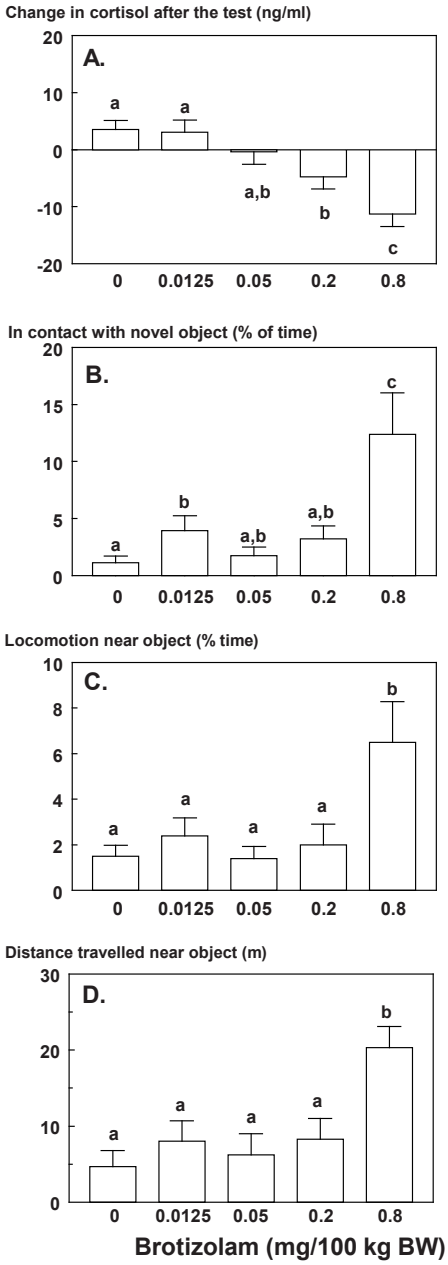

Figure 4.2 Effects of brotizolam (0.0125, 0.05, 0.2 or 0.8 mg/100 kg body weight) or vehicle (0) on plasma cortisol and on behavioral measures recorded in calves during the novel object (NO) phase of the test: A. change in plasma cortisol after termination of the test, i.e., the difference between the cortisol level immediately after the test and that measured 10 min later; B. time spent in contact with the novel object (% of test time); C. time spent in locomotion (% of test time) within a 1.5 m radius from the NO; D. distance travelled (m) withih a 1.5 m radius from the NO. Data are predicted means ± S.E. ^{a,b,c}. Values without a common superscript differ significantly (P<0.05, Fisher's LSD).

Table 4.3 shows those measures that were not significantly affected by brotizolam treatment. Tendencies ($P<0.10$) towards a treatment effect were observed for the heart rate response to introduction of the NO, and for the latencies to enter the OF and to first contact with the NO. Across treatment groups, the latency to first contact with the NO largely changed in parallel with, and inversely to the accumulated time spent in contact with it (Table 4.3, Figure 4.2). The average latency to first contact with the NO in the highest dose group tended to differ ($P<0.10$) from that in the verhicle group. Differences between treatments in the average latency to enter the OF and the average heart rate reponse to the NO did not appear to be dose-related (see Table 4.3 for predicted means).

Table 4.3

Significance (*P*-value) of effects of brotizolam on behavioral and physiological measures (means ± S.E.) recorded in heifer calves during the open field and novel object phases of the test.

Measure	Brotizolam (mg/100 kg body weight)										*P*-value
	0 (vehicle)		0.0125		0.05		0.2		0.8		
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	
Open field test											
Latency to enter test arena[a] (s)	9.1	13.6	50.8	17.7	49.5	18.3	7.7	17.7	35.3	18.4	0.06
Locomotion (% of time)	31.3	4.1	37.3	5.6	35.2	5.7	36.5	5.5	44.0	5.9	0.52
Distance travelled (m)	58.8	10.9	77.4	14.1	63.0	14.5	81.9	14.0	85.1	14.6	0.52
Vocalizations[b]	41.7	11.4	42.0	14.6	25.1	12.0	28.6	12.6	11.1	10.5	0.63
Heart rate start box[c] (beats/min)	41.8	4.7	34.1	6.7	25.4	6.9	36.3	6.7	21.8	6.9	0.13
Heart rate arena[c] (beats/min)	24.1	4.3	22.1	6.2	14.9	6.4	20.9	5.7	13.9	7.1	0.64
Nonmotor heart rate arena[c,d] (beats/min)	25.0	4.5	21.9	6.2	15.0	6.4	20.7	5.8	11.6	7.6	0.55
Nonmotor heart rate arena[c,e] (beats/min)	24.2	4.5	22.1	6.3	15.0	6.7	20.8	6.1	14.0	7.4	0.68
Novel object test											
Latency to first contact with the object[f]	58.3	11.1	70.9	12.5	81.8	9.1	31.5	13.1	26.8	9.7	0.06
Vocalizations[b]	16.7	8.9	23.9	12.8	31.5	10.1	18.5	10.0	29.5	16.0	0.80
Heart rate[c] (beats/min)	25.0	4.7	27.0	6.1	14.3	6.9	37.0	6.7	12.4	6.9	0.07
Nonmotor heart rate (beats/min)[g]	25.0	4.9	27.0	6.3	14.3	7.0	36.9	7.7	12.4	7.2	0.14
Plasma cortisol											
Cortisol immediately after test[h] (ng/ml)	26.8	3.7	20.5	4.7	14.3	4.9	21.3	4.7	31.4	4.9	0.16
Cortisol 10 min after test[h] (ng/ml)	30.4	4.0	23.5	5.1	13.9	5.3	16.6	5.1	20.1	5.3	0.11

[a] Predicted means on original scale. Analysis of variance was performed on log-transformed data.
[b] Analysed as a binary variable. Predicted means for the percentage of calves with 1 vocalization or more.
[c] Response over baseline heart rate.
[d] Predicted means for heart rate corrected for the time spent in locomotion (s) during the open field test.
[e] Predicted means for heart rate corrected for the average speed (m/s) during the open field test.
[f] Analysed as a binary variable. Predicted means for the percentage of calves with a latency above the median.
[g] Predicted means for heart rate corrected for the average speed (m/s) during the novel object test.
[h] Response over pre-test plasma cortisol level.

DISCUSSION

Despite the fact that behavioral tests designed to measure fear responses have been widely used in a variety of domestic animal species (Romeyer and Bouissou, 1992; Boissy and Bouissou, 1995; Keer-Keer et al., 1996; Ruis et al., 2001), limited attention had been given to their stringent validation. Many studies have examined correlational aspects of putatively fear-related behaviors using multivariate statistical techniques such as principal component or factor analysis (Jones et al., 1991; Romeyer and Bouissou, 1992; De Passillé et al., 1995; Kilgour et al., 1996; Vandenheede et al., 1998; Andersen et al., 2000b; Janczak et al., 2002; Viérin and Bouissou, 2003; Müller and Schrader, 2005). Although this is a useful approach, potentially enabling the identification of underlying characteristics or motivational states controlling clusters of behaviors, it does not clearly identify the biological meaning of such underlying factors. Therefore, their interpretation is in essence somewhat arbitrary (Archer, 1973; Weiss et al., 1998). Indeed, based on the outcome of multivariate analyses, different authors can sometimes interpret the same parameters and results in quite different ways. For example, walking and vocalization by (young) bovines in a novel environment were thought to reflect fear in one study (De Passillé et al., 1995), but social motivation in another (Müller and Schrader, 2005). Correspondingly, there is a risk of circularity when certain behavioral measures are *a priori* presumed to represent "reference measures" of fear (Romeyer and Bouissou, 1992; Boissy and Bouissou, 1995; Vandenheede et al., 1998). The inclusion of physiological measures may significantly strengthen assumptions about putative behavioral measures of fear (Boissy, 1995; Jones, 1996), but physiological measures that can be triggered by a state of fear, e.g. adrenocortical activation, may also be influenced by other motivational states (Lebelt et al., 1996). For these reasons, we believe that pharmacological studies (using anxiogenic and/or anxiolytic agents) constitute a vital endpoint in the process of experimental validation of a paradigm intended to measure fear responses in (domestic) animals.

The present pharmacological experiment builds on previous behavioral and physiological work suggesting that the reactivity of calves in OF and NO tests is mediated by multiple characteristics or traits, and that the calves' behavioral interaction with a NO reflects their underlying fearfulness (Van Reenen et al., 2004, 2005). Therein, we proposed that the latency to contact the NO was positively, and the time spent in contact with it was negatively associated with the level of fear (Van Reenen et al., 2005). This idea is clearly supported by our current findings: calves that had received the

highest dose of the anxiolytic brotizolam tended to touch the NO sooner and then spent significantly longer in contact with it than did vehicle-treated ones. In addition, the brotizolam-induced increase in locomotion, in particular locomotion close to the NO, agrees with our previous report that locomotion was positively correlated with the amount of interaction with the NO (Van Reenen et al., 2005). In contrast, neither locomotion during the OF phase nor vocalization during the OF and NO phases were affected by brotizolam. These results are consistent with our earlier suggestion (Van Reenen et al., 2004, 2005) that locomotion and vocalization may reflect characteristics that are independent of fearfulness, for example, activity or coping style, and underlying sociality, respectively (see also Koolhaas et al., 2007). The multifactorial regulation of cattle's reactivity to behavioral tests was also recently suggested following principal component analysis of the OF test responses of adult cows (Müller and Schrader, 2005). Our finding that the effects of brotizolam on the time spent in locomotion and the distance travelled during the NO phase, in comparison with its effects on locomotion close to the NO (within a 1.5 m radius), were less clearly dose-dependent may be the consequence of locomotor activity in a test arena reflecting multiple underlying motivations. Perhaps locomotion exihibited away from the object during the NO phase bears some similarity with locomotion during the OF phase.

We are aware that benzodiazepines can exert sedative side effects, and result in muscle weakness, impaired locomotor coordination or reduced locomotor activity during OF testing (Hughes, 1993; Marin et al., 1997; Andersen et al., 2000a;). However, we consider it unlikely that such effects accounted for the present results. We observed no incidences of ataxia during either of the OF or NO test phases. Moreover, brotizolam did not influence locomotion during the OF phase, but increased it when the NO was present. Therefore, we argue that the present effects are truly anxiolytic, implicating a reduced level of fear as one of the crucial factors underpinning the behavioral changes in brotizolam-treated calves. Similar to findings in various rodent models of unconditioned anxiety (Merali et al., 2003; Ohl, 2003), our data are best explained by the assumption that in the NO test a conflict existed between the motivation to explore the unfamiliar object and that to avoid potential danger. High levels of fear likely inhibited exploratory behaviors, i.e., they attenuated interaction with the NO as well as associated locomotion, whereas administration of the anxiolytic compound brotizolam diminished this inhibition. Since we observed the strongest effects in the highest dose group (0.8 mg/100 kg body weight), it seems that the anxiolytic dose of brotizolam in cattle is higher than the

orexigenic (i.e., appetite increasing) one (0.2 mg/100 kg body weight; see Doll and Dirksen, 1990; Moloney et al., 1990).

Concomitant with anxiolytic behavioral alterations, benzodiazepines generally attenuate stress-induced elevations in circulating glucocorticoids (Lahti and Barsuhn, 1974; De Boer et al., 1990; De Souza, 1990). However, assay of blood samples taken directly after cessation of the combined OF and NO test revealed that brotizolam did not significantly diminish plasma cortisol responses (elevations from baseline) to novelty in our calves. Similarly, acute diazepam administration reduced anxiety-like behaviors of rats in the elevated plus maze (a well validated model of anxiety, see Pellow et al., 1985), but failed to decrease corresponding corticosterone responses (Wilson et al., 2004). These results suggest a dissociation of the anxiety-reducing effects of benzodiazepines and their influence on the hypothalamo-pituitary-adrenal (HPA) axis. A likely factor contributing to this dissociation is the ability of benzodiazepines to increase basal or non-stress levels of ACTH and corticosteroids, in some conditions even to concentrations well within the stress-induced range (Calogero et al., 1990; Kalman et al., 1997; Vargas et al., 2001; Mikkelsen et al., 2005). Evidence suggests that underlying mechanisms may involve the activation of specific subtypes of central $GABA_A$ receptors (i.e., receptors for gamma-amino butyric $acid_A$, the major inhibitory neurotransmitter in the mammalian brain, and the site of action of benzodiazepines) (Rudolph et al., 1999; Mikkelsen et al., 2005), or pathways independent of the $GABA_A$ receptor (Vargas et al., 2001). It is, therefore, quite conceivable that plasma cortisol levels in brotizolam-treated calves were already elevated at the end of the pre-test phase (Table 4.1). Nevertheless, post-test plasma cortisol concentrations decreased at a much faster rate after administration of brotizolam rather than vehicle, thereby corroborating the notion that in our test behavioral anxiolysis was accompanied by less persistent cortisol responses because of lower levels of fear. Kalman et al. (1997) described adrenocortical response patterns in rats subjected to restraint stress that would also fit this explanation: in comparison with the vehicle group, plasma corticosterone levels in diazepam treated animals were higher immediately prior to stress application (60 min after injection), but lower following restraint. Future research needs to determine the more detailed kinetics of adrenocortical activation in calves treated with brotizolam or other types of benzodiazepines.

Collectively, the present endocrine findings reveal the importance of evaluating the persistence of the HPA axis response in benzodiazepine-treated animals rather than relying solely on a single post-stressor sample.

Variations in the dosage of brotizolam affected heart rate responses to the NO corrected for the time spent in locomotion, but there were no significant differences between vehicle and brotizolam-treated calves, and the relationship between the dose of brotizolam and average (nonmotor) heart rates was ambiguous (Figure 4.1, Table 4.3). Likewise, brotizolam only tended to affect the latency to enter the open field arena, and treatment effects on average latencies were not dose-related (Table 4.3). Thus, neither the heart rate response to the NO phase nor the latency to enter the OF can be unequivocally interpreted in terms of fear. This agrees with our previous finding that neither of these measures was significantly correlated with the amount of interaction with a novel object shown by the calves (Van Reenen et al., 2005).

In humans, the concepts of state and trait anxiety have been proposed to differentiate between (1) anxiety that is situation-evoked or experience-related (state), and (2) innate (trait) anxiety, which is an enduring feature of the individual (Lister, 1990). These concepts have also been used to interpret data from laboratory rodents tested in pharmacologically validated paradigms for measuring fear and anxiety responses (Belzung and Griebel, 2001; Ohl et al., 2003). When applied to domestic animals, state anxiety may refer to the level of fear elicited by situational factors related to rearing and husbandry conditions, while trait anxiety is akin to underlying fearfulness, i.e., the propensity to be easily frightened (Boissy, 1995; Jones, 1996). Our previous studies revealed marked consistency over time in the behavioral and adrenocortical responses of individual calves to a NO test (Van Reenen et al., 2004, 2005), thus supporting the existence of stable underlying characteristics, and the suggestion that NO tests can be used to examine an equivalent of trait anxiety in cattle. The use of behavioral tests to evaluate state (fear) and trait anxiety (i.e., fearfulness) is particularly relevant in the general context of farm animal welfare assessment, and, more specifically, for the appraisal of genotypes, housing systems and husbandry practices. However, it remains to be unequivocally demonstrated that our NO test will fully satisfy these objectives.

CONCLUSION

The present study demonstrated anxiolytic effects of the benzodiazepine derivative brotizolam on the behavioral and adrenocortical responses of heifer calves subjected to a test combining exposure to an open field and the eventual introduction of a novel object. Compared to the vehicle treatment, brotizolam increased the times spent in contact with the novel object and in locomotion during that phase of the test. Brotizolam injection also accelerated the post-test decrease in plasma cortisol

concentrations. This anxiolytic agent did not, however, affect vocalization during the OF and NO phases or locomotion during the OF phase of the test. Brotizolam, therefore, specifically influenced those measures that were previously found to be mutually interrelated. Collectively, these findings strongly support our view that the interaction of calves with a NO in a novel arena represents a valid behavioral index of fear and fearfulness, and that vocalization and locomotion in an OF reflect other independent characteristics.

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CHAPTER 5

Individual differences in behavioral and physiological responsiveness of primiparous dairy cows to machine milking

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ABSTRACT

An experiment was performed in primiparous dairy cows (n = 23) to examine consistency of individual differences in reactivity to milking, and correlations between measures of behavior, physiology and milk ejection. Responsiveness to milking was monitored during the first machine milking, on day 2 of lactation, and during milkings on days 4 and 130 of lactation. Measurements included kicking and stepping behavior, plasma cortisol and plasma oxytocin, heart rate, milk yield, milking time, milk flow rate and residual milk obtained after administration of exogenous oxytocin. With repeated early lactation milkings residual milk and the incidence of abnormal milk flow curves decreased. On day 130 of lactation all heifers exhibited normal milk ejection. Except for area under the plasma cortisol against time curve, all measures were consistent over time between day 2 and day 4 of lactation as indicated by significant rank correlations. Individual differences in the the behavioral response to udder preparation were consistent over time between early lactation milkings and day 130 of lactation. Residual milk, milk yield, maximum milk flow rate, plasma oxytocin and heart rate during udder preparation were similarly interrelated on day 2 and day 4 of lactation. High heart rate responses on day 2 and day 4 were associated with enhanced inhibition of milk ejection. In contrast, behavior recorded during the milking process was unrelated to ease of milk removal. Our results indicate that milking at the beginning of lactation may be stressful to some heifers, to the extent that milk ejection is inhibited, but less disturbing to others. The existence of consistent behavioral and physiological responses in the present study suggests that responsiveness of dairy heifers to milking is mediated by stable animal characteristics.

Key words: individual differences, milk ejection, behavior, stress, temperament

INTRODUCTION

In dairy cows, disturbed milk ejection is common particularly during the first milkings of primiparous animals. One likely mechanism underlying this phenomenon involves stress-induced inhibition of oxytocin release (Bruckmaier and Blum, 1998; Tucker, 2000). The milking process constitutes several factors that may be stressful for naive periparturient heifers, including novelty and close interaction with a human handler. Milking cows in an unfamiliar environment has been shown to result in elevated levels of plasma cortisol, higher heart rates, reduced oxytocin release, and lower milk yields concomitant with an increase in the fraction of residual milk (Bruckmaier et al., 1993; Rushen et al, 2001). Reduced milk yields, higher residual milk fractions, and increased heart rates, together with an increase in stepping frequency with the hind legs during milking, have also been observed in cows milked in the presence of a human who had previously handled them aversively (Rushen et al., 1999b).

In addition to environmental conditions, ease of milk removal and responsiveness to milking in heifers may also depend on qualities of the individual animal. Many studies report large (within-treatment) individual variation in behavioral and physiological reactions of dairy cows to milking (e.g., Schams et al., 1984; Bremner, 1997; Rushen et al., 1999b; Tancin et al., 2001). There are two lines of evidence suggesting that such individual differences might reflect the existence of fundamental individual characteristics mediating cow reactivity. First, individual (dairy) cattle display consistent responses over time to treatments that contain aspects of environmental factors which may also be present during milking, such as handling (Kerr and Wood-Gush, 1987; Grandin, 1993) or exposure to novelty (Hopster, 1998). Consistent individual differences in responsiveness to environmental challenge in cattle have been proposed to indicate differences in temperament or fearfulness, which are believed to represent basic personality traits that may be accessible for genetic selection (Boissy, 1995; Grandin, 1997). Second, limited research implies possible relationships directly between personality or temperamental traits and measures of milk ejection. Uvnäs-Moberg et al. (1990) obtained significant correlations between oxytocin levels primiparous women had in response to breast feeding and personality scores of anxiety. Likewise, Lyons (1989) found in dairy goats that stable individual differences in the behavioral expression of temperament (i.e., based on approach and avoidance of humans in test encounters, and behavior in the milking parlor) were associated with differences in residual milk fractions. Similar studies in dairy cows, addressing individual differences in (milking) temperament rather than

differences between treatment groups, have been unable to demonstrate a relationship between parlor behavior and milk yield (Dickson et al., 1970; Purcell et al., 1988). However, these latter studies relied on subjective ratings made by herdsmen in commercial dairy herds, and did not include measures of physiology or milk ejection. Knowledge on behavioral and physiological determinants of (milking) temperament in dairy cows, and on the relationship between temperament and milk production characteristics such as yield and milking speed, is relevant to practical dairy industry, since it may enable the prediction of, for example, ease of milk removal based on other behavioral or physiological variables, and it may help to define a temperamental trait that could be used in genetic selection (see Schutz and Pajor, 2001).

The present experiment aimed to objectively assess, in a uniform group of dairy heifers, milk production characteristics and behavioral and physiological responses to milking, and to examine consistency of individual differences over milkings as well as correlations between measures of behavior, physiology and inhibition of milk ejection. We hypothesized that especially at the beginning of lactation consistent individual differences may reflect dimensions of temperament, since heifers have been suggested to become accustomed to milking procedures somewhere between 7 to 40 days of lactation (Lefcourt and Barfield, 1995; Bremner, 1997). The identification of behavioral or physiological correlates of disturbed milk ejection was also expected to benefit understanding its underlying mechanism. Specifically, we measured heart rate, reflecting activity of the sympathetic nervous system, in conjunction with the release of plasma oxytocin in response to machine-milking, and were, therefore, able to address questions concerning the extent to which inhibition of milk ejection in dairy heifers is mediated by increased sympathetic activity, by central inhibition of oxyocin release, or both (Lefcourt, 1986; Bruckmaier and Blum, 1998).

MATERIALS AND METHODS

The present experiment was carried out at the experimental farm of ID-Lelystad, The Netherlands. The experiment had been approved of by the Institute's Animal Care and Use Committee.

Animals, Housing and Management

Twenty three primiparous Holstein Friesian cows were used, all born and reared at the experimental farm of ID-Lelystad, The Netherlands. During pregnancy the heifers were kept in the same group. One or two days before calving, heifers were transferred to

individual calving pens. After calving, each heifer was housed in a separate straw-littered pen together with her calf, in a barn away from the calving area. The calf remained with the dam at least for the first 36 hours after calving, and was allowed to suckle. Calves were removed at 0900 h on the second day after calving (i.e., on day 2 of lactation; day of calving is day 0), and the heifers stayed in the individual pens until the afternoon when they were machine milked for the first time. Immediately after completion of the first machine milking, heifers entered a dairy herd of 60 cows that was housed in a cubicle barn. All heifers taking part in the present experiment calved over a 4-month period in the same calving season. Except for day 2 of lactation, the heifers were milked twice daily together with the rest of the herd, between 05.15 h and 06.15 h in the morning and between 15.30 h and 16.30 h in the afternoon.

All milkings took place in a fully automated double-three open tandem milking parlor using De Laval milking equipment with electronic milk meters. The milking vacuum was set at 44 kPa. Pulsation rate was 60 cycles/min with a 64:36 pulsation ratio. Clusters were automatically removed when milk flow dropped below 0.2 kg/min. Milking was performed by one of three milkers alternating milking on a 4-day basis. Cows were fed a total mixed diet for ad libitum intake consisting of maize silage, grass silage, concentrates and minerals. In the milking parlor, cows received 1 kg of concentrates at a rate of 100 g per 21 s.

Experimental milkings and observations

Responsiveness to milking was monitored in each heifer on three occasions: during the first machine milking, in the afternoon on day 2 of lactation, during the fifth milking, in the afternoon on day 4 of lactation, and during one afternoon milking between days 110 and 150 of lactation (average: day 130). The distribution of calving dates over time allowed for a maximum of two heifers being tested on the same day.

On each day of testing at 11.00 h, heifers were subjected to a brief clinical inspection by a veterinarian, and an indwelling jugular catheter (1.4 m, polythene, Portex Ltd, UK) was non-surgically inserted via a 12 gauge needle (Intraflon, Vygon, Ecouen, France) and fitted into a custom-made pouch that was glued to the side of the neck of the animal and covered with an elastic neck bandage for further protection. Catheters were filled with citrate solution (0.1 *M* in sterile physiological saline) to prevent blood clotting.

Thirty minutes prior to milking, a baseline blood sample was taken from the catheter, and heifers were fitted with a heart rate monitoring system (Polar Electro Oy,

Helsinki, Finland) which recorded mean heart rates during 5-s intervals (Hopster and Blokhuis, 1994). Heart rate was continuously monitored until the end of milking. To allow undisturbed recording of pre-milking baseline heart rate on day 4 and on day 130 of lactation, heifers were kept in an area of the cubicle house adjacent to the waiting room in front of the milking parlor and separated from the rest of the herd by fences until the beginning of milking. At 15.15 h, the herd was collected in the waiting room and five cows were allowed to enter the milking parlor. Then the experimental heifer was taken individually to the milking parlor through the exit lane running alongside the waiting room, and placed in the remaining milking stall that was adapted for experimental purposes. Next, the milker started preparing the udder of the experimental heifer for one min according to a fixed protocol. Preparation consisted of 20 s of rhythmic and gentle stroking with the back of the right hand on the lower thigh of the left hind leg of the cow (approximately one up-and-down movement of the hand per s), followed by a period of 40 s of udder cleaning with a dry cotton towel. Appropriate timing was provided by an experimenter who was also present in the operating pit during milking, and engaged in collecting blood samples and recording times that marked relevant phases and events in the milking process with the use of a portable computer equipped with the Observer Software System for Behavioral Research (Noldus Information Technology, Wageningen, The Netherlands). Immediately after udder preparation a blood sample was taken, and the milking cluster was attached by the milker. Additional blood samples were taken every minute for the first 10 minutes after cluster attachment, and at 15 and 20 minutes after cluster attachment. Subsequently, 10 IU of oxytocin (Apharmo BV, Duiven, The Netherlands) was administered intravenously through the jugular catheter, and the milking cluster was reattached to obtain residual milk (Bruckmaier et al., 1993; Rushen et al., 2001). Finally, the heifer was released from the milking stall, retrieved by the experimenter at the end of the exit lane, and the heart rate monitor as well as the jugular catheter were removed. In case of a second heifer that needed to be tested, the previously described procedure was repeated from the moment an animal was taken to the milking parlor. Heart rate data were transferred to a personal computer afterwards for further analyses.

Blood was collected into 10-ml evacuated tubes (Vacuette, Greiner BV, Alphen a/d Rijn, The Netherlands) containing heparin. Blood samples were held in ice water, and centrifuged within 1 h after collection for 12 minutes at 2500 g. Plasma was stored at -20 ºC until assayed for cortisol (all samples) and oxytocin (all samples except those taken at 15 and 20 minutes after cluster attachment).

Behavioral responses of heifers to milking were observed by viewing video-taped recordings of milking sessions, obtained with the use of a camera that was mounted in the operating pit opposite to the experimental milking stall. Each instance of kicking or stepping with the hind legs was recorded. A kick was scored whenever a cow forcefully and rapidly moved one of the hind legs towards either the milker or the milking cluster. A step was defined as any other movement of one of the hoofs. Behavioral recordings were analyzed using the Observer software system for behavioral research (Noldus Information Technology, Wageningen, The Netherlands).

Milk yield, maximum milk flow rate and milking time were automatically recorded by the milking system and downloaded to a central computer. The milking glass of the milking stall used for milking experimental heifers was suspended from a digital scales, and cumulative weight of the amount of milk was recorded every 5 s for the duration of milking. These data were processed at a later stage to obtain milk flow curves.

By manipulating the automatic control of the entrance doors of the milking parlor throughout episodes of testing, the milker made sure that experimental heifers remained in the milking stall for the appropriate length of time, and that the remaining milking stalls were occupied by other cows at all times. In order to minimize differences in treatment between heifers, milkers were instructed to behave as standardized as possible towards heifers during experimental milkings, i.e., to refrain from any physical contacts with the animals other than during udder preparation and replacing dislodged cups, and not to use a kick-bar to reduce kicking. During milkings other than the three experimental ones, heifers normally entered the milking parlor via the waiting room together with the rest of the herd, were free to choose any milking stall unless other heifers were tested, and received routine treatment by the milkers.

Hormone assays

Plasma levels of cortisol were determined by a time resolved fluoro immunoassay in unextracted bovine plasma (Erkens et al., 1998). Samples were run in duplicate. The intra-assay coefficients of variation for control samples with concentrations of 71.1, 39.2 and 10.3 $ng.ml^{-1}$ were 8.2, 7.9 and 11.3% (n = 16) respectively. The corresponding inter-assay coefficients of variation were 10.7, 11.4 and 18.4% (n = 20). The lower detection limit for a 20 µl sample was 0.5 ng/ml. Concentrations of plasma oxytocin were analyzed using a radioimmunoassay developed by Schams (1983). Assay sensitivity was 0.3 pg/ml. Intra-assay and inter-assay coefficients of variation were 8 and 11%, respectively. Extraction recovery was over 70%.

Data Processing and Statistical Analyses

Average heart rates were calculated during a 5 min baseline period between five and 10 minutes after starting the heart rate recording, during the one min period of preparation and during machine milking. We hypothesized that sustained increases of heart rate relative to baseline during udder preparation or milk ejection may affect milk ejection. Therefore, we calculated heart rate responses by subtracting baseline values from average heart rates during preparation and during milking, respectively. Kicks and steps were expressed as number/min during preparation and milking, respectively. Since preliminary analyses revealed that the number of kicks was significantly correlated with the number of steps both during preparation and during milking, we decided to consider the number of kicks and the number of steps plus kicks in each phase of the milking process as measures of behavioral responsiveness. For plasma cortisol and plasma oxytocin, integrated areas under the hormone against time after cluster attachment curves were calculated. Residual milk was expressed as a percentage of total milk, where the latter is the sum of the milk yield obtained during milking plus residual milk obtained after administration of exogenous oxytocin.

Milk flow curves (kg milk/min against time) were derived from cumulative weights of the amount of milk recorded during milking, graphically depicted and qualitatively evaluated. Milk flow curves obtained in the present experiment were divided into three categories: those with a complete cessation of milk flow within two minutes after cluster attachment, those with a distinct and transient depression of milk flow within two minutes after attachment (i.e., bimodal curves), and curves not belonging to either of these latter two categories.

Behavioral measures, area under the curve for cortisol and oxytocin, oxytocin after preparation, heart rate responses, residual milk, milk yield, milking time and maximum milk flow rate obtained at each day of lactation were first analysed with an analyses of variance model which included identity of the milker as a fixed effect. For non-normally distributed variables including fractions (i.e., residual milk) and count data (i.e., frequencies of behaviors) the binomial and Poisson variance functions were employed, respectively. The analyses was based on quasi maximum likelihood (McCullagh and Nelder, 1989). None of the variables were significantly affected by the identity of the milker, which was, therefore, not included as a source of variation in subsequent analyses. Differences between sampling times or days in lactation were calculated per animal and analysed with Wilcoxon's matched pairs signed rank test. Tests

on differences were performed to avoid the need for modelling complex dependence structures between data from the same animal. The rank test is based on minimal model assumptions, i.e. the common assumption of a shift alternative. For pairs of variables, observed either within or between days of lactation, Spearman rank correlations were calculated. Principal component analysis (PCA) was employed to analyse and objectively summarize relationships between multiple variables (Jolliffe, 1986). Variables were scaled prior to PCA, i.e., PCA was performed on the Pearson correlation matrix. Principal components produced by PCA are linear combinations of the original variables, and represent condensed new variables reflecting independent characteristics underlying the correlation matrix. The first component explains most of the variance (expressed in terms of the first eigenvalue), the second component explains most of the remaining variation and so forth. The coefficients of the scaled variables, the so-called loadings, indicate the importance of each of the original variables for the principal components. A separate PCA was carried out on the data of each day of lactation. The residual milk fraction (p) was logit-transformed (logit(p) = log(p/1-p)) prior to inclusion in the PCA. Only principal components with eigenvalues larger than one were retained for further analyses.

All statistical calculations were performed with the statistical programming language Genstat (Genstat 5 Committee, 1993).

RESULTS

Differences Between Days of Lactation

On all days of testing, plasma cortisol continuously increased during the first 10 minutes after cluster attachment (Figure 5.1). Cortisol levels at 15 minutes were lower ($P < 0.01$) compared to levels at 10 minutes after cluster placement, and further declined ($P < 0.01$) over the next five minutes. As indicated by large standard errors, response patterns of plasma oxytocin were highly variable among individuals on all days of testing (Figure 5.1). On average, heifers sustained oxytocin levels elevated over baseline until at least eight minutes after cluster attachment (Figure 5.1).

Means and standard errors of milk production characteristics and behavioral and physiological measures on respective days of lactation are presented in Table 5.1. Over days of lactation, the average residual milk fraction progressively decreased, and maximum milk flow rate and oxytocin after preparation increased. Measures of cortisol release decreased on day 4 in comparison with day 2. In contrast to oxytocin after preparation, the integrated amount of oxytocin generated after cluster attachment did not

significantly alter in the course of lactation. Baseline heart rate was substantially lower on day 130 in comparison with day 2 and day 4. Except for heart rate change during milking, behavioral and cardiac responses were generally lowest on day 4 relative to the other days of testing.

Table 5.1.

Milk production characteristics, and behavioral and physiological measures recorded in primiparous cows (n = 23) during milking sessions on days 2, 4 and 130 of lactation.

	Day of lactation					
	Day 2		Day 4		Day 130	
Parameter	Mean	SEM	Mean	SEM	Mean	SEM
Milk yield (kg)	8.4[a,b]	0.88	7.2[a]	0.39	10.6[b]	0.36
Residual milk (% of total milk[1])	34[a]	5.2	18[b]	3.7	9[c]	0.9
Maximum milk flow rate (kg/min)	3.2[a]	0.26	4.2[b]	0.24	4.7[c]	0.28
Duration of milking (s)	372[a]	25.2	275[b]	11.0	333[a]	18.0
Kicks (number/min)						
During preparation	3.0[a]	0.94	1.5[b]	1.00	3.2[a]	0.86
During milking	0.7	0.17	0.7	0.20	0.4	0.08
Steps plus kicks (number/min)						
During preparation	4.4[a]	1.37	1.8[b]	1.10	5.7[c]	1.46
During milking	1.5[a,b]	0.25	1.3[a]	0.31	2.3[b]	0.40
Baseline heart rate (beats/min)	97.6[a]	1.73	98.1[a]	1.32	82.4[b]	1.56
Heart rate change[2] (beats/min)						
During preparation	12.0[a]	1.60	4.6[b]	1.01	9.7[a]	1.25
During milking	8.3[a]	0.82	5.8[a,b]	1.39	4.2[b]	1.13
Oxytocin after preparation (pg/ml)	11.4[a]	2.66	15.0[a,b]	3.33	27.8[b]	5.57
Oxytocin[3]	92.4	11.73	113.7	16.36	116.0	13.61
Cortisol[3]	174.6[a]	12.99	134.5[b]	17.35	138.2[a,b]	13.08
Cortisol 10 minutes after attachment (ng/ml)	11.5[a]	0.78	8.9[b]	0.96	9.6[a,b]	1.01

[1]Total milk (kg): milk yield plus residual milk obtained after administration of exogenous oxytocin

[2]Relative to baseline

[3]Area under time after cluster attachment versus hormone curve

[a,b,c]Means in the same row without a common superscript differ significantly (P <0.05)

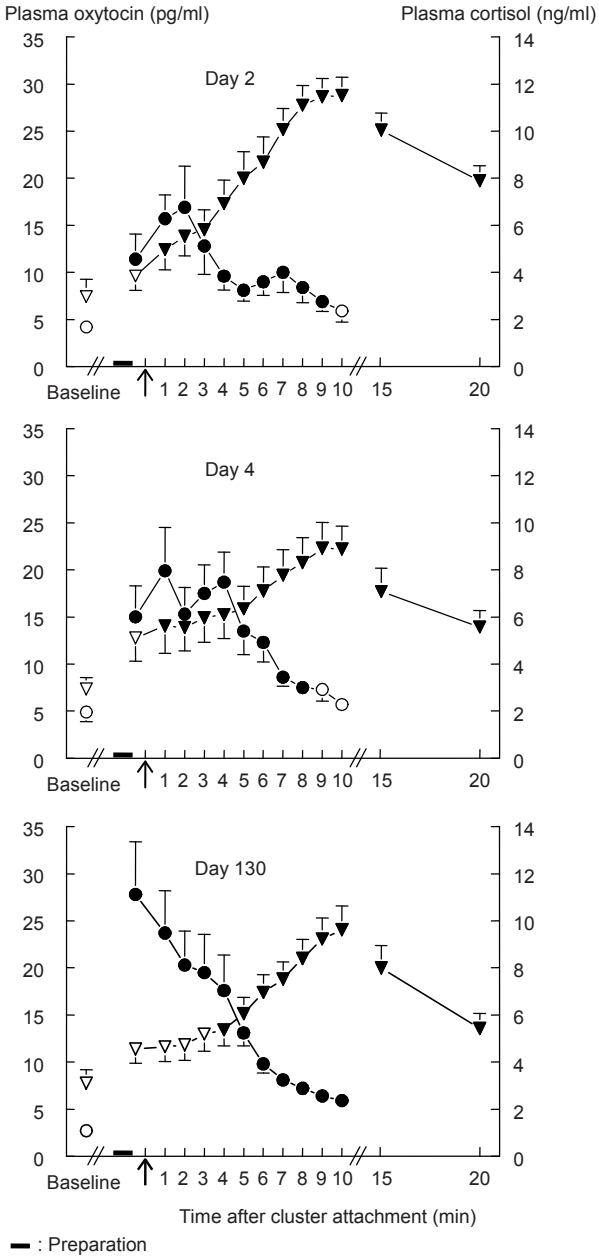

Figure 5.1. Mean (± SEM) plasma concentrations of oxytocin (circles) and cortisol (triangles) in primiparous dairy cows during milking sessions on days 2, 4 and 130 of lactation. Circles and triangles are closed when values differed significantly ($P < 0.05$) from baseline values. Arrow below X-axis indicates the time of cluster attachment.

On day 2 of lactation, milk flow curves of the first (early cessation of milk flow) and second (bimodal) category were observed in 2 and 8 heifers, respectively. On day 4, curves of the first category were no longer present, and 2 heifers exhibited curves of the second category. On day 130 of lactation, all milk flow curves were of the third category.

Consistency of Measures over Time and Correlations between Variables

With the exception of milk yield, oxytocin after preparation and area under the cortisol against time curve, individual differences in all measures considered in the present report were consistent over time between day 2 and day 4 of lactation (Table 5.2). Individual differences in the the behavioral response to udder preparation were consistent over time between early lactation milkings (day 2 and day 4) and day 130 of lactation (Table 5.2).

Measures of cortisol release were not significantly correlated with behavioral measures or with measures of heart rate response. Only on day 2 and day 4, number of kicks during preparation was significantly correlated with heart rate change during preparation (rank correlations of 0.44 and 0.43 on day 2 and day 4, respectively, $P < 0.05$). Number of kicks during milking significantly correlated with heart rate change during milking on day 4 (rank correlation 0.62, $P < 0.01$), but not on the other days of testing.

Individual differences in the behavioral response to preparation (i.e, kicks, and steps plus kicks) were not significantly associated with individual differences in the behavioral response to milking. In contrast, heart rate change during preparation was positively correlated with heart rate change during milking (rank correlations of 0.57, 0.73 and 0.73 on day 2, day 4 and day 130, respectively, $P < 0.01$).

Table 5.2.

Spearman rank correlations over intervals between days 2, 4 and 130 of lactation, for milk production characteristics, and behavioral and physiological measures recorded during milking in primiparous cows (n = 23).

	Interval between days of lactation		
Parameter	Day 2 - 4	Day 2 - 130	Day 4 - 130
Residual milk (% of total milk)	0.62**	0.27	0.06
Maximum milk flow rate (kg/min)	0.38†	0.45*	0.41†
Duration of milking (s)	0.40†	0.31	0.46*
Kicks (number/min)			
During preparation	0.82**	0.45*	0.66**
During milking	0.42*	0.10	0.15
Steps plus kicks (number/min)			
During preparation	0.83**	0.39†	0.56**
During milking	0.46*	0.04	0.31
Baseline heart rate (beats/min)	0.68**	- 0.08	- 0.21
Heart rate change[1] (beats/min)			
During preparation	0.59**	- 0.33	- 0.21
During milking	0.52*	- 0.08	- 0.17
Oxytocin[2]	0.70**	0.28	0.21
Cortisol 10 minutes after attachment (ng/ml)	0.40†	0.22	0.27

[1]Relative to baseline

[2]Area under the time after cluster attachment versus oxytocin curve

† $P < 0.10$

* $P < 0.05$

** $P < 0.01$

Neither any behavioral measure, nor any measure of plasma cortisol release was significantly correlated with the residual milk fraction. Residual milk, milk yield, maximum milk flow rate, oxytocin and heart rate during preparation were interrelated on day 2 and day 4 of lactation (Table 5.3). The patterns of correlations between variables presented in Table 5.3 could be adequately summarized using principal component analyses (PCA). PCA successively performed on data recorded on day 2 and day 4 of lactation, produced in each case a single component with eigenvalue larger than 1. Eigenvalues were 3.11 and 2.74, and percentages of total variance explained by the first principal

component were 62 and 55% on day 2 and day 4, respectively. On day 130, PCA resulted in two components with eigenvalues larger than 1 (1.91 and 1.37), explaining 38 and 27% (65% in total) of the variation. Figure 5.2 shows the distribution of the variables included in the PCA in relation to the first two components. Both on day 2 and day 4, heart rate during preparation and residual milk loaded positively, and milk flow, milk yield and oxytocin loaded negatively on the first principal component (Figure 5.2). Therefore, this latter component was interpreted to reflect inhibition of milk ejection, with heifers having high component scores showing enhanced inhibition of milk ejection, and heifers having low component scores showing reduced inhibition of milk ejection. Importantly, individual differences in component scores of the first principal component obtained on day 2 of lactation significantly predicted individual differences in component scores of the first principal component obtained on day 4 (rank correlation 0.66, $P < 0.01$), evidencing consistency over early lactation milkings of a multivariate response profile associated with milk ejection. There were no significant relationships between component scores of the first principal component on day 2 or day 4, and measures of behavior or cortisol release.

The pattern of interrelated responses that was present on day 2 and day 4 of lactation was markedly changed on day 130. In contrast to day 2 and day 4, the residual milk fraction on day 130 was unrelated to oxytocin and tended to correlate positively with milk yield (Table 5.3), and exclusively loaded on the second principal component (Figure 5.2). In addition, also contrary to day 2 and day 4, heart rate during preparation on day 130 was significantly positively correlated with maximum milk flow rate (Table 5.3), and, together with oxytocin, represented a group of closely interrelated variables constituting the first principal component (Figure 5.2). There were no significant correlations between any principal component score obtained on day 130 and scores of the first principal component derived after PCA on day 2 and day 4, respectively.

Table 5.3

Spearman rank correlations[1] between milk production characteristics, heart rate and oxytocin recorded in primiparous cows (n = 23) during milking sessions on days 2, 4 and 130 of lactation

Measure	1	2	3	4
1. Milk yield (kg)				
2. Residual milk (% of total milk)	- 0.81**			
	- 0.37†			
	0.38†			
3. Maximum milk flow rate (kg/min)	0.52*	- 0.62**		
	0.37†	- 0.36†		
	0.08	0.15		
4. Heart rate during preparation[2]	- 0.47*	0.36†	- 0.36†	
	- 0.34	0.47*	- 0.55**	
	0.12	0.00	0.46*	
5. Oxytocin[3]	0.68**	- 0.82**	0.54**	- 0.49*
	0.37†	- 0.72**	0.34	- 0.40†
	0.30	- 0.05	0.46*	0.29

[1]Within each cell of the matrix, the correlations in the first, second and third row represent the correlations between measures obtained on days 2, 4 and 130 of lactation, respectively

[2]Change relative to baseline (beats/min)

[3]Area under time after cluster attachment versus oxytocin curve

† $P < 0.10$

* $P < 0.05$

** $P < 0.01$

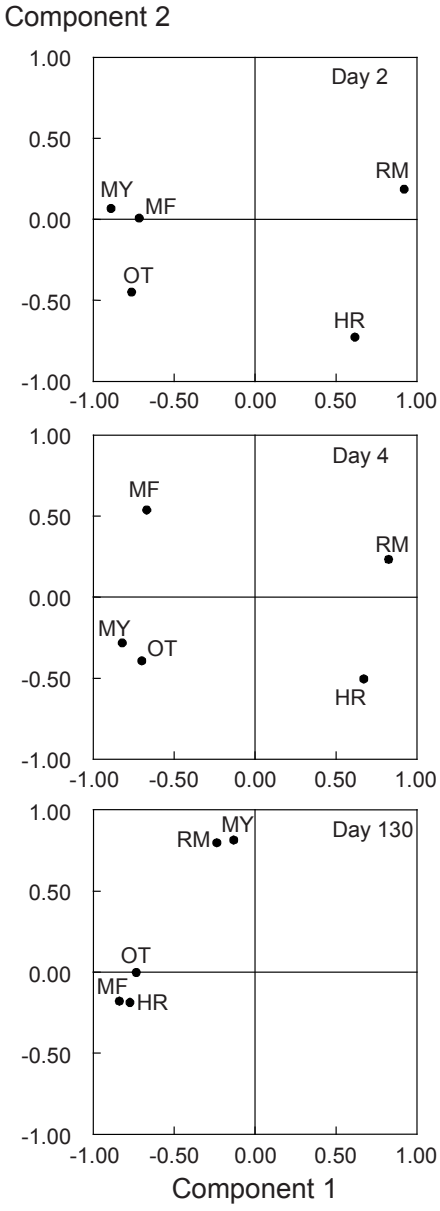

Figure 5.2 Distributions in relation to the first two principal components extracted after principal component analyses (PCA) of variables obtained during milkings of heifers on days 2, 4 and 130 of lactation, with loadings of each variable on the first and second principal component serving as coordinates on the X-axis and Y-axis, respectively. Labels of variables: MY = milk yield; MF = maximum milk flow rate; OT = integrated area under the plasma oxytocin against time after cluster attachment curve; HR = heart rate change during udder preparation relative to baseline; RM = residual milk fraction.

DISCUSSION

The main finding of the present study is that individual heifers showed consistent behavioral and physiological responses to standardized milkings at the beginning of lactation. This strongly suggests that responsiveness of dairy heifers to milking is mediated by stable animal characteristics. Not only individual differences in separate measures proved consistent over early lactation milkings, but also differences in an aggregate measure composed of a weighted combination of interrelated variables, putatively reflecting inhibition of milk ejection. This would indicate that multiple variables were consistently mediated by the same underlying characteristic or mechanism across milkings.

The pattern of correlations on day 2 and day 4 of lactation reported here, fits the mechanism of stress-induced inhibition of milk ejection that has been found in cows milked in an unfamiliar environment (Bruckmaier et al., 1993, Rushen et al., 2001). Residual milk fraction was highly negatively correlated with oxytocin released after cluster attachment, providing evidence for inhibition of oxytocin secretion in those heifers with reduced milk letdown. The coherent interrelationships between measures of milk ejection and heart rate change during preparation, with high heart rate responses associated with enhanced inhibition of milk ejection, suggest the involvement of activation of the sympathetic nervous system in the mechanism underlying perturbed milk removal in our heifers. Increased sympathetic activity, and the release of catecholamines, may result in peripheral inhibition of milk ejection through interference with oxytocin efficacy at the level of the mammary gland (Lefcourt, 1996). This mechanism is usually distinguished from central inhibition of milk ejection by suppression of oxytocin release (Bruckmaier and Blum, 1998; Tucker, 2000). The present data, however, suggest a possible link between increased sympathetic activity and central inhibition of milk ejection. In agreement with our findings, Lefcourt et al. (1997) demonstrated an inverse relationship between noradrenalin concentrations prior to milking and oxytocin responses in dairy ewes, and proposed that peripheral concentrations of noradrenalin should be considered a marker of sympathetic input to hypothalamic mechanisms in control of oxytocin release. Measuring heart rate simultaneously with plasma catecholamines would be a necessary step to examine whether a similar mechanism operates in periparturient heifers during milking.

The sympathetic nervous system may also represent a possible connection between inhibition of milk ejection and basic features of personality such as

temperament or fearfulness. In cattle, both novelty and exposure to people can elicit an emotional state of fear which is characterized physiologically by increased activity of the sympathetic nervous system, and is assumed to be modulated by underlying personality traits defining the vulnerability of an individual to respond (Boissy, 1995; Grandin, 1997). Thus, it is suggested that sympathetic activity prior to and during milking may have been highest in the most fearful heifers, causing the greatest disturbances in oxytocin release and milk removal. Given the relevance in the present study of heart rate change during preparation, when there is close contact between human and cow, fear of people might be important in this respect. In support of a relationship between fearfulness and inhibition of milk removal are studies in dairy goats (Lyons, 1989) and breast feeding women (Uvnäs-Moberg et al., 1990), showing high residual milk fractions in goats fearful of people, and low suckling-induced oxytocin levels in women with high anxiety scores.

Behaviors during preparation were not correlated with behaviors during milking. This means that individual heifers responded differently to different phases in the milking process, and may reflect the difference between preparation and milking in the degree of human contact. The incidence of leg movements in dairy cows, including steps and kicks, occuring in the close presence of the milker has been hypothesized to be positively associated with the level of fear of humans and, on the assumption of a negative fear-productivity relationship, to be negatively associated with milk yield (Hemsworth et al., 1989, Breuer et al., 2000). However, in our experiment there were no relationships in heifers between behavioral measures recorded in the milking parlor and residual milk or an aggregate measure of milk ejection, despite significant (although mostly moderate) correlations between the incidence of kicking and heart rate change during preparation or milking. This finding seems to confirm previous studies in dairy cattle, showing no relationship between parlor behavior and milk yield (Dickson et al., 1970; Purcell et al., 1988). Moreover, Purcell et al. (1988) failed to establish a relationship between the distance individual cows kept to a stationary human, a behavioral measure which is assumed to reflect the level of fear of humans (Hemsworth et al., 1989; Rushen et al., 1999b; Breuer et al., 2000), and their behavior in the milking parlor. Taken together, results in dairy cows appear to contrast with results in dairy goats that revealed significant interrelations between avoidance behavior in the presence of a human, behavior in the milking parlor and residual milk (Lyons, 1989).

To explain the lack of association in dairy cows between behavior and measures of milk ejection or milk yield, Rushen et al. (2001) suggested that different behavioral and physiological responses may be affected independently by different aspects of the

milking process. This suggestion would be plausible if, for example, our results would have pointed to inhibition of milk ejection primarily due to the novelty associated with machine milking, and variation in behavioral responses occuring mainly during periods of interaction with a human. However, both the heart rate response that appeared to be implicated in the inhibition of milk removal and the behavioral response that was not related to measures of milk ejection, were recorded during the same phase of the milking process, i.e., udder preparation. Bremner (1997) proposed that there is a non-linear relationship in heifers between tameness and behavioral reactivity, with both very tame and very fearful animals being less likely to step or kick during milking, which could provide an alternative explanation for the absence of significant (linear) correlations between measures of behavior and milk ejection. However, on the assumption that heifers with the most disturbed milk removal at the beginning of lactation were the most fearful ones and vice versa, this explanation does not seem to apply to our findings since non-linear patterns of association between behavioral responses and measures of milk ejection could not be found (results not shown). Thus, both heifers with enhanced and reduced inhibition of milk ejection were equally likely to consistently exhibit either high or low levels of leg movements. Therefore, we postulate that during early lactation milkings different heifers expressed the same underlying tendency, probably fearfulness, similarly from a physiological point of view, i.e., by an inhibition of oxytocin release proportionate to the level of fear, but differentially in terms of behavior, i.e., by performing a variable but individually stable number of leg movements. In line with this suggestion, Boissy (1995) argued that an emotional state of fear may be accompanied by various, sometimes even contradictory, patterns of behavior in the range between active avoidance (i.e., flight, escape) and immobility. Variability in behavioral patterns during milking among putatively non-fearful heifers, i.e., animals with reduced inhibition of milk removal, may originate from individual differences in (the expression of) temperamental characteristics other than fearfulness which have also been suggested to mediate cow behavior during milking, such as agressiveness or boldness (Purcell et al., 1988; Rushen et al., 1999b). The possibility of differential behavioral but similar physiological responses to fear-evoking stimuli during milking in dairy cows is supported by data from Knierim and Waran (1993) showing increased heart rates and reduced milk yields, but no significant behavioral changes, when the same cows were milked by relief milkers instead of the usual milkers.

The present experiment provided little evidence for consistency over milkings in measures of cortisol release, or for the existence of relationships between measures of

cortisol release and other variables. These findings confirm previous studies in support of inhibition of oxytocin release independent of cortisol (Mayer and Lefcourt, 1987; Marnet and Negrao, 2000), but also suggest that plasma cortisol may not be very useful to characterize individual differences in stress responsiveness in the context of milking. The feasability of plasma cortisol as a sensitive indicator of stress-induced activation of the hypothalamo-pituitary adrenal axis during milking is probably hampered by the fact that (machine) milking of dairy cows as such stimulates the release of cortisol, which is required for maintenance of lactation (Tucker, 2000). In fact, patterns of milking-induced cortisol release in the present study were similar to the patterns observed by Gorewit et al. (1992) in normal multiparous cows, with peak levels of cortisol 10 min after cluster attachment. Long-lasting elevations of plasma cortisol with maximum levels some 45 min after the beginning of milking were found by Rushen et al. (2001) in dairy cows milked individually in a novel environment, and may be a reflection predominantly of the effect of social isolation since our heifers were milked in the presence of other cows.

Disturbances in milk removal seemed to disappear fairly quickly across early lactation milkings in our study, as illustrated by large decreases in the number of abnormal milk flow curves and in the average residual milk fraction on day 4 relative to day 2 of lactation. This agrees with the study of Bremner (1997) where the incidence of heifers with disturbed milk letdown was found to decline by at least 50% over the first week of lactation. Likewise, during the sixth milking in a series of successive milkings of cows in an unfamiliar environment, milk flow and milk yield were normalized and plasma cortisol was decreased in comparison with the first milking, suggesting a rapidly progressing process of adaptation to environmental challenge (Bruckmaier et al., 1996). Correspondingly, we found lower levels of plasma cortisol with repeated early lactation milkings, together with lower heart rate responses and lower average numbers of leg movements. This latter finding would suggest that adaptation to milking may involve a reduction of all leg movements regardless of the underlying emotional state (e.g., fearfulness, agressiveness, boldness etc.).

On day 130 of lactation, the average residual milk fraction was 9%, which is a level that is characteristic of normal and undisturbed milk ejection (Bruckmaier and Blum, 1998). The pattern of correlations between various measures was fundamentally changed on day 130 relative to day 2 and day 4, since residual milk and heart rate during preparation no longer counteracted milk yield, milk flow rate and oxytocin. Instead, heart rate was positively correlated with measures of milk ejection, and residual milk was unrelated to oxytocin. This latter result has been interpreted as a finding confirming the

theory that beyond a certain threshold level, any additional oxytocin has no further effect on milk removal (Schams et al., 1984; Bruckmaier and Blum, 1998). Notably, individual differences in the behavioral response to preparation, but not to milking, were consistent over time between early lactation milkings and day 130 of lactation, which agrees with long-term consistency in the behavioral reaction of heifers to being touched by a person as reported by Kerr and Wood-Gush (1987). Thus, individually stable behavioral patterns were clearly maintained beyond early lactational stages, but they were no longer accompanied by physiological disturbances due to stress on day 130 of lactation. In the case of previously fearful heifers, a process of learning may have taken place, whereby a persistent behavioral response to a fearful situation is gradually associated with reduced aversiveness, and eventually becomes detached from physiological signs of fear. This mechanism has been proposed to explain the observations that defecation and urination in dairy cows during mildly aversive treatments by handlers decreased as the treatment was repeated, and that residual milk was not affected by the presence during milking of the aversive handler, even though distance scores (i.e., behaviorally expressed levels of avoidance of a human) remained high (Munksgaard et al., 2001).

CONCLUSIONS

Individual primiparous dairy cows responded consistently to milking at the beginning of lactation, both behaviorally and physiologically. Our results indicate that milking at the beginning of lactation may be stressful to some heifers, to the extent that milk ejection is inhibited, and less disturbing to others. Consistent individual differences in responsiveness of heifers to milking may point to the existence of basic temperamental traits mediating reactivity to challenge in individual cows. In contrast to heart rate, behavior recorded during the milking process was unrelated to ease of milk removal in heifers, emphasizing the need for a multivariate approach in defining a desired (milking) temperament.

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CHAPTER 6

Behavioural and physiological responses of heifer calves to acute stressors: long-term consistency and relationship with adult reactivity to milking

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ABSTRACT

The present study investigated the long-term consistency of individual differences in dairy cattles' responses in tests of behavioural and hypothalamo-pituitary-adrenocortical (HPA) axis reactivity, as well as the relationship between responsiveness in behavioural tests and the reaction to first milking. Two cohorts of heifer calves, Cohorts 1 (*N* = 25) and 2 (*N* = 16), respectively, were examined longitudinally from the rearing period until adulthood. Cohort 1 heifers were subjected to open field (OF), novel object (NO), restraint, and response to a human tests at 7 months of age, and were again observed in an OF test during first pregnancy between 22 and 24 months of age. Subsequently, inhibition of milk ejection and stepping and kicking behaviours were recorded in Cohort 1 heifers during their first machine milking. Cohort 2 heifers were individually subjected to OF and NO tests as well as two HPA axis reactivity tests (determining ACTH and/or cortisol response profiles after administration of exogenous CRH and ACTH, respectively) at 6 months of age and during first lactation at approximately 29 months of age. Principal component analysis (PCA) was used to condense correlated response measures (to behavioural tests and to milking) within ages into independent dimensions underlying heifers' reactivity. Heifers demonstrated consistent individual differences in locomotion and vocalisation during an OF test from rearing to first pregnancy (Cohort 1) or first lactation (Cohort 2). Individual differences in struggling in a restraint test at 7 months of age reliably predicted those in OF locomotion during first pregnancy in Cohort 1 heifers. Cohort 2 animals with high cortisol responses to OF and NO tests and high avoidance of the novel object at 6 months of age also exhibited enhanced cortisol responses to OF and NO tests at 29 months of age. Measures of HPA axis reactivity, locomotion, vocalisation and adrenocortical and behavioural responses to novelty were largely uncorrelated, supporting the idea that stress responsiveness in dairy cows is mediated by multiple independent underlying traits. Inhibition of milk ejection and stepping and kicking behaviours during first machine milking were not related to earlier struggling during restraint, locomotor responses to OF and NO tests, or the behavioural interaction with a novel object. Heifers with high rates of OF and NO vocalisation and short latencies to first contact with the human at 7 months of age exhibited better milk ejection during first machine milking.

This suggests that low underlying sociality might be implicated in the inhibition of milk ejection at the beginning of lactation in heifers.

Keywords: Dairy cattle, Individual differences, Temperament, Development, Fearfulness, Coping style, Sociality, HPA axis, Behavioural test, Principal component analysis

INTRODUCTION

There is compelling evidence that many animals, including humans, display consistent individual differences in their behavioural and physiological responses to a variety of challenges. Such consistent differences are thought to reflect the existence of fundamental underlying traits or dimensions, which are at least partly inherited and may affect the individuals' adaptive capacities, health, and life outcomes. Characteristics of this sort have been generally referred to as, for example, fearfulness (Boissy, 1995; Jones, 1996), temperament (Goldsmith et al., 1987; Clarke and Boinski, 1995), coping style (Mason, 1984; Koolhaas et al., 1999), or behavioural syndrome (Sih et al., 2004a). A related term is personality, which is commonly assumed to encompass multiple biologically-based traits or dimensions (Zuckerman, 1991; Caspi, 2000; Erhard and Schouten, 2001; Funder, 2001; Gosling, 2001).

Concepts of individual differences are highly relevant to domestic animal production, since their application may eventually facilitate the improvement of health and coping abilities in farm animals through, for example, selective breeding or individually adjusted husbandry practices (Faure and Mills, 1998; Burrow, 1997; Erhard and Schouten, Jones and Hocking, 1999; 2001; Bolhuis et al., 2003; Boissy et al., 2005; Jones and Manteca, 2009). Thus, there is an increasingly large body of research concerning individual differences in farm animal species, in particular studies reporting the use of various short-term tests to assess behavioural and physiological responses to controlled experimental stressors. Two types of data have supported the notion that individual differences in the reactivity of farm animals to behavioural tests may indicate the existence of broad, constitutional traits or dimensions rather than stimulus-specific characteristics. Firstly, consistent differences have been found, although not universally, over time and across different experimental challenges in various farm animal species, including poultry (Jones, 1988; Miller et al., 2006), pigs (Hessing et al., 1994; Thodberg et al., 1999; Ruis et al., 2000; Janczak et al., 2003b; Bolhuis et al., 2004), and cattle (Boissy and Bouissou, 1995; Hopster, 1998; Schrader, 2002; Müller and Schrader, 2005; Kilgour et al., 2006; Müller and Von Keyserlingk, 2006; Gibbons et al., 2009a). Secondly,

individual differences in stress responsiveness to short-term tests are associated with differences in reactivity or in the function of other biological systems such as the immune and metabolic systems (for salient examples in pigs and cattle, see Hessing et al., 1995; Voisinet et al., 1997a, b; Fell et al., 1999; Petherick et al., 2002; Bolhuis et al., 2003; Geverink et al., 2004; Müller and Von Keyserlingk, 2006), as well as in the predisposition to develop abnormal or stereotypic behaviours (see Redbo, 1998; Geverink et al., 2003; Rodenburg et al., 2004; Jensen et al., 2005, for examples in poultry, pigs and cattle). Apparent relationships between juvenile responses to acute stressors and the characteristics of mature behavioural or physiological reaction patterns in adulthood are also important here. For example, behavioural and physiological reactions of juvenile or prepubertal female pigs to brief exposure to humans and novelty reliably predicted later aspects of reproductive success and maternal behaviour (Spinka et al., 2000; Thodberg et al., 2002a, b; Janczak et al., 2003a). Similarly, individual differences in the behavioural expression of temperament in juvenile goats (based on approach/avoidance of humans) were significantly related to the ease of milk ejection during milking when adult (Lyons, 1989). Collectively, these findings not only point to the early establishment and long-term consistency of underlying characteristics, but also agree with the notion that stable temperamental traits or dimensions in (farm) animals, as in humans, are part of the relatively enduring biological make-up of the individual, and may therefore exert an influence well beyond the biological and developmental context they were originally recorded in (Goldsmith et al., 1987; Caspi, 2000; Goldsmith and Lemery, 2000; Rothbart et al., 2000).

Previously, we showed that heifer calves exhibit consistent individual differences in their behavioural and physiological responses to tests including brief exposure to an open field (OF) and a novel object (NO) test between the ages of 3 weeks and 6-7 months, suggesting that reactivity of calves to challenge is mediated by stable underlying characteristics (Van Reenen et al., 2004, 2005). Consistency of individual differences in reactivity of adult dairy cows to an OF test across successive lactations was demonstrated by Hopster (1998) and Müller and Schrader (2005). However, long-term consistency of individual differences in responsiveness to OF and NO tests from infancy into adulthood has not been studied so far in dairy cattle. Moreover, there is limited information on relationships between responsiveness of dairy cows to behavioural tests and their biological responses in other contexts. Some studies examined OF responses or approach/ avoidance of humans in adult lactating cows as possible correlates of milk production and behaviour in the milking parlour (Kovalcikova and Kovalcik, 1982/83;

Purcell et al., 1988; Uetake et al., 2004), but the actual milk production level and other characteristics related to cow management and physiology (e.g. lactational stage, feeding regime, production group, social rank, etc.) might have confounded these relationships. Hopster et al. (1998) reported a promising association in dairy heifers between the adrenocortical response to an OF test during the first lactation and the immune response pattern to an endotoxin challenge during the next one.

The present study aimed to examine long-term consistency of individual differences in behavioural and physiological reactivity of dairy calves to behavioural tests, including OF and NO tests. Therefore, calves tested at the age of 6-7 months (Van Reenen et al., 2004, 2005) were subsequently re-tested as adult heifers, either during first pregnancy or first lactation. Two tests of hypothalamo-pituitary-adrenal (HPA) axis function were also applied in the same longitudinal fashion. A second objective was to evaluate the relationship between behavioural responses of calves to a range of tests (involving exposure to an OF, a NO, human contact and restraint; see Van Reenen et al., 2004), and later reactivity of the same animals to milking at the beginning of the first lactation. A previous study (Van Reenen et al., 2002) revealed a marked consistency of individual differences in behavioural and physiological responses, including stepping and kicking in the milking parlour and ease of milk letdown, to early lactation milkings. The current design constituted a truly prospective approach, thereby allowing us to robustly test the hypothesis that adaptive capacities in dairy cows are controlled by stable underlying temperamental characteristics.

MATERIALS AND METHODS

The present experiment was carried out at the experimental farm of the Animal Sciences Group of Wageningen University and Research Centre in Lelystad, The Netherlands. The experiment was approved by the Institute's Animal Care and Use Committee.

Animals, housing and management

Two cohorts of heifer calves (Cohort 1 and Cohort 2) were examined longitudinally from the rearing period until adulthood. Both cohorts were reared on the same experimental farm, according to regular feeding and husbandry standards for replacement heifer calves (see Van Reenen et al. 2004, 2005). Two animals had to be culled in Cohort 1 (Van Reenen et al. 2004) so 23 heifers were available for observations during first lacatation. In Cohort 2, 4 of the original 20 calves (Van Reenen et al., 2005)

were culled prior to first lactation, so 16 subjects were available for longitudinal observations.

Between approximately 6 and 13 months of age, all heifers were kept on pasture. Thereafter, they were housed indoors in a cubicle house. All heifers were fed a total mixed diet consisting of maize silage, grass silage, concentrates and minerals. Diet composition was appropriately adjusted according to gestation and lactation stage. First inseminations were carried out at an average age of 15 months. Cohort 1 heifers were kept as a separate group during pregnancy, entered a dairy herd of 60 cows after calving, and were then milked twice a day in a double-three open tandem milking parlour (see Van Reenen et al., 2002). After grazing on pasture, Cohort 2 heifers entered the farm's herd of inseminated heifers, and became part of the collective dairy herd after calving. They were milked twice a day in a large rotary parlour.

During behavioural and hypothalamo-pituitary-adrenal (HPA) axis reactivity testing (see below), heifers under observation were kept in a smaller batch in an area of the cubicle house separated from the rest of the herd by fences.

Observations and data

An overview of the observations recorded in Cohorts 1 and 2 is provided in Table 6.1. Observations during the rearing period were previously performed as part of experiments where calves were repeatedly subjected to the same set of behavioural and physiological challenges from the age of 3 weeks onwards (Van Reenen et al., 2004, 2005). For the present study, examining long-term consistency of responsiveness, we used only the last recording obtained between 6-7 months of age.

Behavioural and physiological responsiveness to milking was monitored in each individual heifer on days 2, 4 and 130 of lactation in a previous study (Van Reenen et al., 2002, but for present purposes only observations collected on day 2 of lactation (the first machine milking) were considered.

Table 6.1

Observations recorded in two cohorts of heifers during longitudinal research from the rearing period up to the first lactation

		Cohort	
Stage in life	Age at testing	1 (*N* = 25)[a]	2 (*N* = 16)
Rearing period	6 - 7 months	Behavioural responses to open field, human, novel object and restraint tests[b]	Behavioural and physiological responses to open field and novel object tests. Pituitary-adrenocortical responses to ACTH and CRH challenge tests[c]
Gestation	22 - 24 months	Behavioural response to open field test	
First lactation	25 - 29 months	Behavioural and physiological responses to milking during the first machine milking on day 2 of lactation[d]	Behavioural and physiological responses to open field and novel object tests. Pituitary-adrenocortical responses to ACTH and CRH challenge tests

[a]Of the original group of 25 calves, 23 animals were available during first lactation.

[b]Data from Van Reenen et al. (2004).

[c]Data from Van Reenen et al. (2005).

[d]Data from Van Reenen et al. (2002)

Tests performed in our previous studies. At 29 weeks of age Cohort 1 calves were individually subjected to four behavioural tests; these involved measuring the calves' behavioural responses to a human (human test), brief isolation in a novel arena or open field (OF test), exposure to a novel object (NO test), and tethering restraint (restraint test). Cohort 2 calves were exposed to OF and NO tests at 26 weeks of age (Van Reenen et al., 2004, 2005). Briefly, in the human test the latency of a calf to make physical contact with a stationary experimenter and the time spent in contact with the experimenter were recorded in a test arena during a 10 min period. In the OF test, each Cohort 1 calf was individually confined in a start box for 3 min before a door was opened and the experimenter briefly touched its lower back to ensure that it immediately entered the OF arena (Van Reenen et al., 2004). Cohort 2 calves were allowed to voluntarily enter the OF (Van Reenen et al., 2005). The time spent in locomotion and the number of

vocalisations were recorded over a 10 min period. In the NO test each calf was individually exposed for 10 min to an unfamilar tambourine connected to either a coloured plastic ball (Cohort 1) or a plastic container (Cohort 2) in a straw-littered test arena. Behavioural measures included the latency to contact the NO, the times spent in locomotion and in contact with the NO, and the number of vocalisations. In the restraint test, calves were tethered for 10 min with a rope attached to a halter, and the time spent moving (any movement of head and/or legs) was recorded.

In addition to their behavioural responses, the plasma cortisol and heart rates of Cohort 2 calves to OF and NO tests were recorded. In addition, HPA axis reactivity tests to exogenous ACTH and CRH were performed in the home pen (see Van Reenen et al., 2005).

Behavioural and physiological challenge tests during gestation and first lactation. Cohort 1 heifers were individually tested in the OF test at the beginning of the third trimester of gestation; (based on gestation stage, 3 batches of 5, 10, and 10 animals, respectively, were created over time). Heifers were tested batch-wise and in a random order. At the time of OF testing the overall average number of days in gestation was 189 (standard error 9.2 days). Like our earlier procedures (Van Reenen et al., 2005), heifers were individually confined in a start box connected to one corner of a 6 m x 6 m test arena with a concrete floor and 2 m high wooden walls, and allowed access to it through remotely controlled, pneumatically-operated swing doors. Each heifer was allowed to voluntarily enter the OF after which the doors were closed behind it. OF tests took place between 09.00 and 12.00 am, and a maximum of 5 heifers was tested on a single day.

The same OF test was used for Cohort 2 heifers at about 100 days into their first lactation, together with a NO test as well as ACTH and CRH challenge tests. All tests and observations were in close accordance with procedures previously applied to the same animals during rearing (Van Reenen et al., 2005). Cohort 2 heifers were divided according to lactation stage into two batches of 7 and 9 animals, respectively, and subsequently studied batch-wise in a random order. At the time of the first test (OF), the number of days into lactation was 98 ± 4.3 (mean ± standard error). Each heifer was exposed to behavioural and HPA axis challenge tests over a 4 day period. For logistical reasons 3 to 5 heifers were tested on a single day. Heifers of the same batch were kept in a separate area of the cubicle house from 12 days prior to the first test until completion of the last one. In the week preceding actual data collection heifers were fitted with a halter to facilitate handling and were habituated to the test conditions on three occasions

(see below). Table 6.2 shows the time schedule of experimental procedures and measurements. The NO test was carried out in a 4.5 m x 4.5 m straw-littered test arena with 2 m high wooden walls. The NO (tambourine + blue plastic container measuring 25 x 25 x 50 cm) was introduced into the arena 3 min after the heifer's entrance. All OF and NO tests were videotaped and scored using the Observer Software System for Behavioural Research (Noldus Information Technology, Wageningen, The Netherlands). During OF and NO tests the number of vocalisations and the time spent in locomotion were recorded. Additional behavioural measures in the NO test included latency to first contact with the object and time spent in contact with it (see Van Reenen et al., 2005 for detailed definitions of behavioural measures). Heart rate was continuously monitored during a period of baseline recording in the home pen 3 days before the OF test (Table 6.2), and during the OF and NO tests with the use of a non-invasive heart rate monitoring system (Polar Electro Oy, Helsinki, Finland) adapted for use in bovines (Van Reenen et al., 2005). Mean heart rates were recorded at 5 second intervals. Before and after each NO and OF test, a blood sample was taken by jugular puncture. On the evening before the ACTH challenge test (Table 6.2) an indwelling jugular catheter was nonsurgically inserted, placed inside a custom-made pouch attached to the animal's neck and covered with an elastic bandage for protection. Catheters were filled with a citrate solution to prevent blood clotting, and rinsed daily to maintain patency (see Van Reenen et al., 2005). During HPA axis reactivity tests, heifers were loosely tethered inside adjacent cubicles and catheters were accessible for blood sampling. In the ACTH challenge test heifers received an intravenous injection of 0.016 IU $ACTH_{1-24}$ (Synacthen)/kg body weight$^{0.75}$. Blood samples were obtained at 15 min before (-15) and at 10, 20, 30, 45, 60 and 75 min after administration of ACTH. In the CRH challenge test a dose of 0.03 µg bovine CRH/kg body weight was given intravenously (see Van Reenen et al., 2005). Blood samples were taken at the same time points as in the ACTH challenge test, as well as at 90 and 120 min after CRH administration. Unlike the procedure at 6 months of age, we refrained from running control HPA axis reactivity tests (vehicle treatment) during lactation since our earlier findings clearly demonstrated that injection of vehicle did not elicit a pituitary-adrenocortical response over baseline (Van Reenen et al., 2005). Blood samples were collected in 10 ml evacuated tubes containing EDTA, kept on ice, and centrifuged for 12 min at 3000 x g at 4°C. Plasma samples were stored at either -20°C until assay for cortisol (all samples), or at -80°C until assay for plasma ACTH (duplicates obtained in CRH challenge tests). Plasma concentrations of cortisol and ACTH were determined as previously described (Van Reenen et al., 2005).

For cortisol, intra-assay and corresponding inter-assay coefficients of variation assessed at high and low cortisol levels ranged between 8 and 11%, and 6 and 10%, respectively. The lower detection limit was 0.5 ng/ml. Intra-assay and inter-assay coefficients of variation for the ACTH assay were 4% and 3%, respectively, at intermediate levels of ACTH. The detection limit was 1 pg/ml. Each of 3 habituation sessions carried out on successive days (Table 6.2) involved the tethering of each heifer for 2 hours in a cubicle, with the heart rate belt and neck bandages used during HPA axis challenge testing attached.

Table 6.2

Timing of experimental procedures employed in Cohort 2 heifers during their first lactation

Day	Experimental procedure
-2 to 0	Habituation to testing conditions
1	Recording of baseline heart rate between 13.00 and 15.00 pm
4	Open field test between 13.00 and 15.00 pm, insertion of jugular catheter
5	ACTH challenge test between 09.00 and 11.00 am
6	Novel object test between 13.00 and 15.00 pm, rinsing of catheter with citrate solution
7	CRH challenge test between 09.00 and 11.00 am, removal of catheter

Responsiveness to first milking. Measurements in Cohort 1 heifers during the first machine milking on day 2 of lactation included kicking and stepping behaviour, plasma oxytocin, heart rate, milk yield, milk flow rate and residual milk obtained after administration of exogenous oxytocin (see Van Reenen et al., 2002).

Data processing and statistical analyses

Data processing and analysis comprised three parts: (1) re-analysis of data previously obtained, (2) processing of data recorded during gestation and first lactation, and (3) analysis of the comparisons and the relationships between data recorded during rearing and those obtained during gestation and first lactation. All statistical analyses were performed using the statistical programming language GenStat (Genstat Committee, 2000).

Re-analysis of data previously obtained during rearing. Previously, data recorded during the rearing period in both calf cohorts were subjected to a principal component analysis (PCA) (Jolliffe, 1986; Van Reenen et al., 2004, 2005). Since the present study was designed to examine long-term consistency of individual differences

the same analyses were repeated using only those calves that were tested both during rearing and first pregnancy or first lactation, i.e. 23 and 16 animals for Cohorts 1 and 2, respectively (see Table 6.1). Table 6.3 shows the varimax-rotated loadings on the first four factors of 8 behavioural measures recorded in 23 Cohort 1 heifers at the age of 7 months.

Table 6.3

Loadings[a] on the first four factors extracted by principal component analyses (PCA), after varimax rotation, of behavioural measures recorded in Cohort 1 calves at the age of 7 months (*N* = 23), eigenvalues and proportions of total variation explained by each factor

Measures	Factor 1	Factor 2	Factor 3	Factor 4
In contact with human (% of time)	- 0.18	0.04	0.07	**0.93**
Latency to first contact with human (s)	**0.80**	- 0.06	- 0.10	- 0.40
Locomotion during OF[b] test (% of time)	- 0.01	**0.91**	- 0.05	0.24
Vocalisations during OF test (number)	**- 0.87**	0.19	0.06	0.01
Locomotion during NO test (% of time)	- 0.05	**0.91**	- 0.07	- 0.17
In contact with NO (% of time)	0.03	- 0.09	**0.97**	0.07
Latency to first contact with object (s)	- 0.29	0.21	- 0.26	0.29
Vocalisations during NO test (number)	**- 0.84**	- 0.14	- 0.22	0.08
Eigenvalues	2.96	1.77	1.23	0.84
Variance explained	37%	22%	15%	11%

%, percentage; s, seconds

[a]Loadings greater than 0.50 are indicated in bold

[b]OF, open field; NO, novel object

This loading pattern was highly similar to that previously obtained in the full cohort of 25 calves (Van Reenen et al., 2004). The first three factors had eigenvalues greater than 1, and were used for further analysis. The first factor had high loadings for vocalisations during OF and NO tests and a high, but opposite, loading for the latency to first contact with the human. Locomotion during the OF and NO tests and the time spent in contact with the NO exclusively loaded on factors 2 and 3, respectively (Table 6.3). Factors 1 – 3 were labelled: (1) “Vocalisation”, (2) “Locomotion”, and (3) “Interaction with a novel object”. PCA of behavioural and physiological measures recorded in 16 Cohort 2 heifers during rearing resulted in a two-component solution identical to that previously obtained in the full cohort of 20 calves (Van Reenen et al., 2005). Locomotion during the OF test loaded exclusively on the second principal component (Figure 6.1). The first principal component had high negative loadings for latency to first contact with the NO and the cortisol responses to the OF and NO tests, and a high positive loading for time spent in contact with the NO (Figure 6.1). The first and second principal components explained 56% and 21% of the total variance, respectively. The first principal component was assumed to reflect underlying fearfulness, i.e. the propensity to be easily frightened (Jones, 1996; Van Reenen et al., 2005).

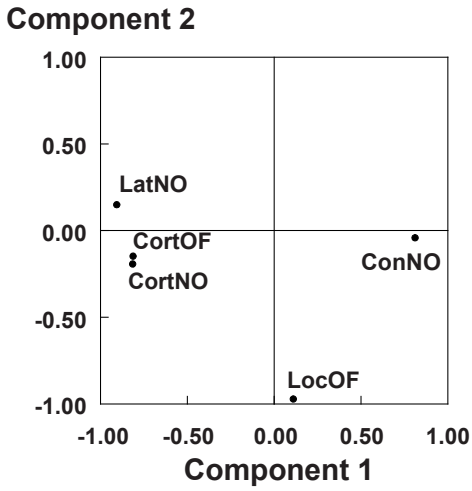

Figure 6.1 Distributions in relation to the first two principal components extracted after principal component analyses (PCA) of variables obtained in Cohort 2 heifer calves at the age of 6 months (*N* = 16) during an open field test and a novel object test. Loadings of each variable on the first and second principal component serve as coordinates on the X-axis and Y-axis, respectively. Labels of variables: ConNO, In contact with novel object; LatNO, Latency to first contact with novel object; LocOF, Locomotion during open field test; CortNO, Cortisol response to novel object test; CortOF, Cortisol response to open field test.

Averages and standard errors of behavioural and physiological measures recorded during rearing in Cohort 2 calves were re-calculated for the 16 animals that were followed up until adulthood.

Behavioural and physiological challenge tests during gestation and first lactation. The accumulated times spent in locomotion, and in contact with the NO were expressed as percentages of the total test duration.

In Cohort 2 heifers, average heart rates were calculated for a 10-min baseline period between 5 and 15 min after starting the baseline heart rate recording, for the 3-min period after the animal was confined in the start box, for 10 min after the swing doors were opened (OF test) and for 10 min after the NO was lowered (NO test). Heart rate responses were calculated by subtracting baseline values from average heart rates during confinement in the start box, and during the OF and NO tests, respectively. Cortisol responses to the OF and NO tests were calculated by subtracting pre-test from post-test plasma cortisol levels. Plasma cortisol and ACTH responses to the ACTH or CRH challenge test were summarized by calculating integrated areas under the 'hormone against time after administration of exogenous ACTH or CRH' curves. As a measure of adrenocortical sensitivity to endogenous ACTH, the cortisol/ACTH ratio after injection was calculated by dividing the area under the plasma cortisol against time curve by the area under the plasma ACTH against time curve (Van Reenen et al., 2005).

Unlike our statistical analysis of data obtained during the rearing period (Van Reenen et al., 2005), we refrained from PCA of behavioural and physiological measures recorded in lactating Cohort 2 heifers at 29 months of age. At that age more than half of the animals totally ignored the novel object and the remaining ones hardly paid any attention to it (see below). Thus, the latency to first contact with the NO and the time spent in contact with it lacked meaningful variation, and could not be included in a PCA. Interaction with the NO was therefore transformed into a single discrete (binary) characteristic, with values "0" (no interaction with the NO) or "1" (interaction with the NO).

Potential batch effects on behavioural measures (Cohorts 1 and 2), except interaction with the NO, and physiological ones (Cohort 2) were examined with analysis of variance using models with batches entered as levels of a fixed effect (i.e., with three and two levels in Cohorts 1 and 2, respectively). Percentages of test time were analysed with a logistic regression model with a multiplicative overdispersion factor, where the variance function is a multiple of the binomial variance function. Numbers of vocalisations (i.e. count data) were analysed as overdispersed Poisson data on a logarithmic scale. Analyses of logistic and log linear models were based on maximum

quasi likelihood, with overdispersion parameters estimated from Pearson's generalized chi-square statistic (McCullagh and Nelder, 1989). No significant differences (*F*-test in analysis of variance and quasi likelihood ratio tests in logistic and log linear models) were found between batches. Therefore, batch was not included as a source of variation in subsequent analyses.

The relationships between interaction with the NO and other dependent measures were examined with an analysis of variance model that included interaction with the NO as a fixed effect with two levels (contact with the NO versus no contact with the NO). The methodology was identical to that used for the analysis of the effects of batches (see above).

Correlations between pairs of measures were calculated using the non-parametric Spearman rank correlation test (Conover, 1980).

Responsiveness during milking. Measures recorded during first lactation in Cohort 1 heifers were previously summarized with the use of PCA (Van Reenen et al., 2002). The first principal component extracted after PCA of plasma oxytocin released during milking, heart rate during preparation, milk yield, average milk flow rate during milking, and residual milk obtained after administration of exogenous oxytocin explained 66% of the total variance, and was suggested to represent a composite index of inhibition of milk ejection. Heart rate during udder preparation and residual milk loaded positively, and milk flow, milk yield and oxytocin loaded negatively on the first principal component (Van Reenen et al., 2002). Behavioural measures recorded during the milking process included the numbers of kicks and steps plus kicks (Van Reenen et al., 2002). There was no relationship between these behavioural measures and measures of milk ejection (Van Reenen et al., 2002).

Analysis of differences between ages within animals. Differences within the same test between ages (rearing period versus gestation or first lactation) were compared non-parametrically using the Wilcoxon matched-pairs signed rank test (Conover, 1980).

Relationships between calf and cow data. Relations between measures obtained during rearing and those obtained during gestation or first lactation were assessed using the Spearman rank correlation (Conover, 1980). In Cohort 1 animals, we correlated behavioural measures obtained during rearing as well as scores of principal components from a PCA of these variables (see Table 6.3) with OF behaviours observed during gestation, and with scores of the composite index of inhibition of milk ejection (see section above on Responsiveness during milking). Likewise, in Cohort 2 animals

behavioural and physiological measures recorded during rearing were correlated with those obtained during lactation. We specifically examined the relationships between, on the one hand, measures that were thought to be associated with underlying fearfulness during rearing, including the first principal component extracted after PCA of adrenocortical and behavioural responses to novelty (see Figure 6.1), and on the other, heart rate reactivity to confinement in the start box and cortisol responses to OF and NO tests during lactation. Associations between interaction with the NO at 29 months and measures obtained during rearing were analysed using analysis of variance according to the method previously described (see 2.3.2.1.).

RESULTS

Cohort 1

Differences in levels of measures between ages. At 22 months of age, heifers voluntarily entered the OF arena in 4.0 ± 0.6 s (mean ± standard error). Locomotion in the OF test was similar during rearing (25.4 ± 1.6% of total time, S.E. 1.60; Van Reenen et al., 2004) and at 22-months (22.1 ± 1.62%), but fewer ($P < 0.001$) vocalisations were recorded at 22 (2.9 ± 0.95) than at 7 months (12.5 ± 2.2; Van Reenen et al., 2004). At 22 months, 10 of the 25 heifers failed to vocalize during the OF test.

Relations between measures across time. Significant correlations were apparent between behavioural variables measured during rearing and OF behaviours during gestation (Table 6.4). Individual differences in locomotion during the OF and NO tests during rearing, as well as scores of the corresponding factor labelled “Locomotion”, reliably predicted individual differences in locomotion during the OF test at 22 months of age. Locomotion at 22 months was also significantly correlated with the time spent moving the head or legs (struggling) during the 7-month restraint test. A significant but modest correlation was apparent between the number of OF vocalisations at 7 and at 22 months of age. No significant relationships were found between OF vocalisations at 22 months and vocalisations during NO tests, and scores of the factor labelled “Vocalisation” at 7 months. Similarly, there were no significant correlations between scores of the factors labelled “Interaction with a novel object”, “Interaction with a human” and OF behaviours during gestation.

Table 6.4

Significant Spearman rank correlations (N = 25) between measures obtained in Cohort 1 heifer calves at the age of 7 months, and locomotion and vocalisations during an open field test recorded in the same animals during gestation at the age of 22 months

	Behaviour during open field test at 22 months	
Measures at 7 months	Locomotion (% of time)	Vocalisations (number)
Locomotion during OF[a] test (% of time)	0.48*	
Locomotion during NO test (% of time)	0.55*	
Vocalisations during OF test (number)		0.42*
Movement during restraint test (% of time)	0.76***	0.50*
Scores of "Locomotion" factor[b]	0.63**	

[a]OF, open field; NO, novel object.

[b]Factors were extracted by principal component analysis (PCA), after varimax rotation, of eight behavioural measures. High scores of the "Locomotion" factor indicate high levels of locomotion during OF and NO tests

* P <0.05

** P <0.01

*** P <0.001

Of all behavioural measures obtained during rearing, only the numbers of vocalisations during the OF and NO tests and scores of the "Vocalisation" factor were correlated with the composite index of inhibition of milk ejection (Table 6.5). These correlations indicate that animals showing high levels of vocalisation and short latencies to make contact with the human during behavioural testing at 7 months of age exhibited better milk ejection on day 2 of lactation. The correlation between the inhibition of milk ejection and the number of OF vocalisations at 22 months of age was non-significant (Spearman rank correlation -0.38, P < 0.10, N = 23). Behavioural measures recorded during rearing or gestation were not correlated with kicking and stepping behaviour observed during first milking.

Table 6.5

Spearman rank correlations (N = 23) between behavioural measures obtained in Cohort 1 heifer calves at the age of 7 months and scores of the first principal component extracted by principal component analyses (PCA) of variables recorded in the same heifers during milking on day 2 of lactation

	Measure during lactation
Measures at 7 months	Scores of first principal component[b]
Vocalisations during OF[a] test test (number)	- 0.55**
Vocalisations during NO test (number)	- 0.66**
Scores of "Vocalisation" factor[c]	0.51*

[a]OF, open field; NO, novel object.

[b]PCA involved five interrelated variables associated with the ease of milk letdown: heart rate during udder preparation, residual milk fraction, plasma oxytocin released during milking, maximum milk flow rate, and milk yield. The first principal component served as an aggregate measure of inhibition of milk ejection. High scores indicate enhanced inhibition of milk ejection

[c]Factors were extracted by PCA (principal component analyes), after varimax rotation, of eight behavioural measures recorded in 7-month-old heifer calves during three behavioural tests. Loadings and scores were recalculated using only the 23 heifers examined both at 7 months of age and during first lactation. See Table 6.2 for loadings of behavioural measures on factors. The "Vocalisation" factor refers to Factor 1 in Table 6.2, with high negative loadings for the number of vocalisations during the open field and novel object tests, and a high positive loading for the latency to first contact with the human in the human test

* $P < 0.05$

** $P < 0.01$

Cohort 2

Differences in levels of measures between ages. At 29 months of age, heifers vocalised less often during the OF and NO tests than at 6 months (Table 6.6). This decrease over time was most pronounced for the NO test. Relative to the rearing period, lactating heifers largely ignored the novel object (Table 6.6); 9 of the 16 heifers did not contact the object at all at 29 months. The time spent in locomotion and the heart rate response to the NO were significantly lower at 29 than at 6 months. In contrast, cardiac and locomotor responses to the OF test were similar at both ages (Table 6.6).

The adrenal output of cortisol in response to exogenous ACTH and CRH, and the pituitary output of ACTH after CRH challenge were substantially higher during lactation

than during rearing (Table 6.6). At 29 months of age, heifers exhibited more persistent plasma ACTH and cortisol responses to HPA axis challenge tests than at 6 months of age, with higher peak values and longer times required for hormones to return to baseline values (Figure 6.2). However, the cortisol/ACTH ratio after injection of CRH was not affected by age (Table 6.6). This implies similar adrenocortical sensitivities over time. Baseline cortisol levels obtained prior to ACTH and CRH challenge were systematically higher at 29 months of age than at 6 months (Figure 6.2). During HPA axis challenge testing all cows appeared calm and most of them lay down and ruminated. There were no age effects on plasma cortisol responses to the OF and NO tests (Table 6.6).

Relations between measures at 29 months of age. At 29 months of age, the correlation between the cortisol response to the OF and NO tests approached significance (rank correlation 0.48, $P < 0.10$). The heart rate response to confinement in the start box was moderately positively correlated with the cortisol responses to the OF and NO tests (rank correlations 0.46 and 0.54, respectively, $P < 0.10$ and $P < 0.05$). There were no associations between interaction with the novel object and any other variables at 29 months (analysis of variance with interaction with the novel object introduced as a fixed effect with two levels: contact with the object versus no contact with the object). Measures of locomotion during the OF and the NO tests were not correlated. Individual differences in the number of vocalisations, on the other hand, were consistent across OF and NO tests (rank correlation between tests 0.67, $P < 0.01$). Measures of vocalisation, locomotion and adrenal responsiveness to OF and NO tests were mutually uncorrelated ($P > 0.10$ for all possible correlations), suggesting that locomotion, vocalisation and cortisol responses to OF and NO tests represented independent characteristics.

There were no significant relationships between measures of HPA axis reactivity to exogenous ACTH or CRH and any other behavioural or physiological measures, except between cortisol after CRH challenge (area under the curve) and the cortisol response to the NO test (rank correlation 0.54, $P < 0.05$).

Table 6.6

Behavioural and physiological measures (mean ± S.E.) recorded in Cohort 2 heifers (*N* = 16) during open field and novel object tests, and in ACTH and CRH challenge tests at the ages of 6 and 29 months

Measure	Age			
	6 months		29 months	
	Mean	S.E.	Mean	S.E.
Open Field Test				
Latency to enter the open field arena (s)	10	1.9	6	0.3
Locomotion (% of time)	24.2	2.17	22.5	1.63
Vocalisations (number)	15.1[a]	2.83	8.1[b]	2.14
Plasma cortisol after test[1] (ng/ml)	11.4	2.25	13.2	2.67
Heart rate in startbox[2] (beats/min)	34.0	3.47	28.6	3.81
Heart rate during test[2]	15.3	2.78	13.5	2.07
Novel Object Test				
Locomotion (% of time)	11.8[a]	1.40	7.9[b]	1.23
In contact with novel object (% of time)	1.3[a]	0.33	0.1[b]	0.04
Latency to first contact with the object (s)	184[a]	57.4	407[b]	65.4
Vocalisations (number)	11.3[a]	2.82	2.3[b]	1.02
Plasma cortisol after test[1] (ng/ml)	8.0	1.38	9.3	2.08
Heart rate during test[2] (beats/min)	19.9[a]	3.31	11.3[b]	2.52
ACTH challenge test				
Plasma cortisol[3]	220.0[a]	22.42	964.6[b]	39.4
CRH challenge test				
Plasma ACTH[3]	806.4[a]	98.19	2818.0[b]	318.95
Plasma cortisol[3]	993.4[a]	60.78	4200.4[b]	249.79
Ratio between cortisol and ACTH[4]	1.44	0.14	1.59	0.15

[a,b]Different superscripts within a row indicate a significant difference ($P < 0.05$) between ages

[1]Response over corresponding pre-test plasma cortisol level

[2]Response over baseline heart rate recorded at the same age

[3]Area under hormone against time after administration of ACTH or CRH curve

[4]Area under plasma cortisol against time curve divided by area under plasma ACTH against time curve

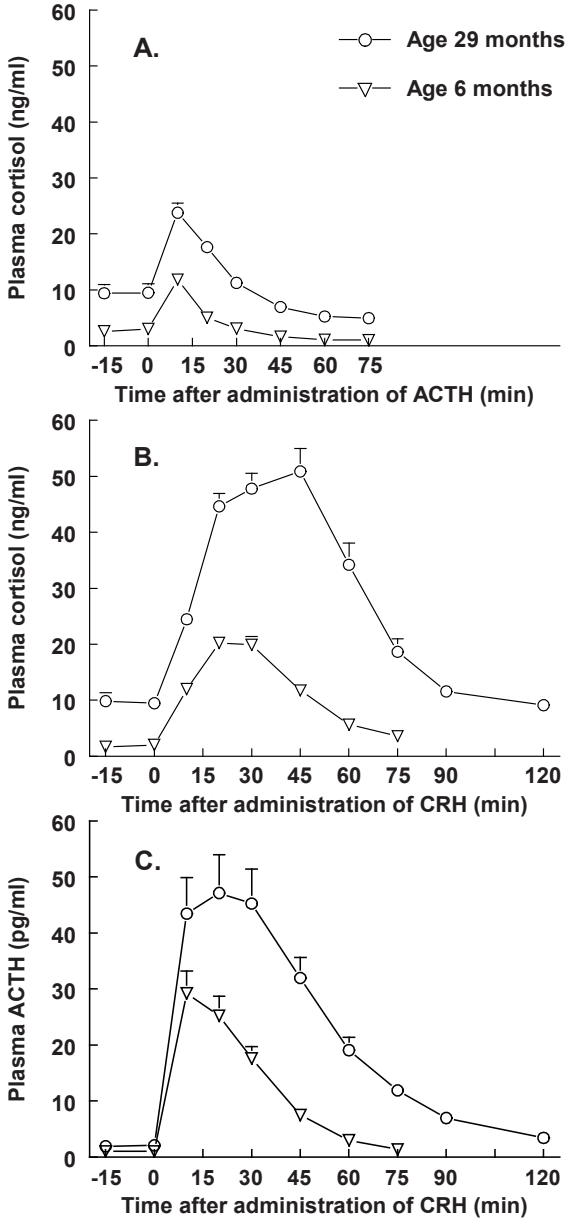

Figure 6.2 Reactivity of the hypothalamo-pituitary-adrenal axis (mean ± S.E.) to exogenous ACTH or CRH in Cohort 2 heifers at the ages of 6 and 29 months: A. plasma cortisol after administration of 0.016 I.U./kg0.75 ACTH1-24; B. plasma cortisol after intravenous administration of 0.03 µg/kg bovine CRH; C. plasma ACTH after intravenous administration of 0.03 µg/kg bovine CRH.

Relations between measures across time. Table 6.7 presents a matrix of correlations between measures obtained at 6 months of age that were assumed to be associated with underlying fearfulness (Van Reenen et al., 2005, 2008) and cortisol responses to OF and NO tests at 29 months of age. Individual differences in scores of the principal component summarizing adrenocortical and behavioural responses to novelty at 6 months of age reliably predicted individual differences in cortisol responses to OF and NO tests at 29 months. The same was largely true for individual differences in the respective variables constituting the first principal component, i.e. the cortisol responses to the OF and NO tests, the latency to first contact with the novel object and the time spent in contact with it at 6 months of age (Table 6.7). In addition, locomotion during the NO test at 6 months was negatively correlated with the cortisol response to the OF test at 29 months (rank correlation -0.53, $P < 0.05$), and the latency to first contact with the novel object at 6 months was positively correlated with the heart rate response to confinement in the start box at 29 months of age (rank correlation 0.52, $P < 0.05$). The heart rate response to confinement in the start box was significantly correlated between ages (rank correlation 0.66, $P < 0.01$). There were no significant relationships between interaction with the novel object at 29 months of age and any other behavioural or physiological measure recorded during rearing.

Individual differences in locomotion and vocalisation during OF and NO tests were largely consistent over time (Table 6.8). With the exception of the number of vocalisations during the NO test at 6 months and the time spent in locomotion during the NO test at 29 months, there were no significant correlations between locomotion and vocalisation across ages (Table 6.8). The time spent moving during the OF test and vocalisations during the OF and NO tests at 6 months of age were not significantly correlated with the cortisol responses to the OF and NO tests at 29 months. Locomotion and vocalisation during OF and NO tests at 29 months were unrelated to adrenocortical responses to OF and NO tests at 6 months of age.

Apart from the cortisol response to CRH other measures of HPA axis reactivity to ACTH or CRH challenge were not correlated across ages (rank correlation 0.52, $P < 0.05$).

Table 6.7

Significant Spearman rank correlations (N = 16) between measures obtained in Cohort 2 heifer calves at the age of 6 months, and measures obtained in the same animals at the age of 29 months

	Measures at 29 months of age	
Measures at 6 months of age	Cortisol[a] after OF[b] test	Cortisol after NO test
Latency to contact novel object (s)	0.75**	
In contact with novel object (% of time)	- 0.56*	
Cortisol after NO test (ng/ml)	0.77**	0.53*
Cortisol after OF test (ng/ml)	0.54*	0.51*
Scores of first principal component[c]	- 0.81**	- 0.50*

[a]Cortisol after each test is a response over the corresponding pre-test plasma cortisol level

[b]OF, open field; NO, novel object

[c]Principal components were extracted by principal component analyses (PCA) of five variables recorded in 6-month-old calves. See Figure 6.1 for loadings of variables on the first and second principal component. High scores of the first principal component indicate low cortisol responses to the novel object test and to the open field test, short latencies to first contact with the novel object, and high percentages of time spent in contact with the novel object

* P <0.05

Table 6.8

Significant Spearman rank correlations (N = 16) between locomotion and vocalisations during open field and a novel object tests recorded in Cohort 2 heifer calves at the age of 6 months, and the same measures obtained in the same animals at the age of 29 months

	Measures at 29 months			
Measures at 6 months	Locomotion during OF[a] test	Locomotion during NO test	Vocalisations during OF test	Vocalisations during NO test
Locomotion during OF test (% of time)	0.71**			
Locomotion during NO test (% of time)	0.50*	0.55*		
Vocalisations during OF test (number)			0.83***	0.59*
Vocalisations during NO test (number)		0.54*	0.52*	

[a]OF, open field Test; NO, novel object

* P <0.05

** P <0.01

*** P <0.001

DISCUSSION

To the best of our knowledge, the present report is the first to show long-term consistency of individual differences in behavioural and adrenocortical responses to OF and NO tests from the rearing period until adulthood in dairy cattle. Our findings clearly support the idea that the responsiveness of dairy cows to challenging situations/events is governed by developmentally stable underlying characteristics.

Our previous studies in calves showed that locomotion, vocalisation and interaction with a novel object during OF or NO tests were largely uncorrelated, and that cortisol responses to OF and NO tests were specifically related to interaction with the novel object, but with no other behavioural measure (Van Reenen et al., 2004, 2005). Therefore, we proposed that the reactivity of calves to OF and NO tests is mediated by multiple independent traits related to locomotion, vocalisation, and correlated adrenocortical and behavioural responses to novelty, respectively (Van Reenen et al., 2004, 2005). Since locomotion, vocalisation, and the adrenocortical responses to NO and OF tests were for the most part uncorrelated, not only within the (young) adults but also between ages, the present results, documenting longitudinal follow-up observations in the same animals, support this suggestion. Moreover, like the rearing period, the numbers of vocalisations during OF and NO tests were significantly inter-correlated at 29 months of age.

In line with our previous work (Van Reenen et al., 2004, 2005), we hypothesize that the rate of vocalisation reflects underlying sociality (or sociability), i.e. the motivation of individuals to remain close to conspecifics (Gibbons et al., 2010). Although there is ongoing discussion about the interpretation of behavioural responses of animals, including bovines, to exposure to OF and NO tests (e.g., Archer, 1973; Munksgaard and Jensen, 1996; Rushen, 2000; Forkman et al., 2007), vocalisation during social isolation is thought to represent an important behavioural determinant of this trait in poultry, sheep and bovines (Jones and Mills, 1999; Watts and Stookey, 2000; Erhard and Schouten, 2001). Indeed, the number of vocalisations expressed by heifers during an OF test was positively correlated with the level of non-aggressive social interactions with group mates in the home pen (Boissy and Bouissou, 1995). Likewise, Lansade et al. (2008) reported that individual differences in the frequency of neighing were stable over time and across isolation and separation tests in horses and, importantly, were also significantly correlated with differences in the latency to reinstate contact with companions during a so-called runway test, which is viewed as a “gold standard” test of social motivation (Mills

and Faure, 1990; Gibbons et al., 2010). Based on these results, it was suggested that the reactivity of horses to social isolation and separation is controlled by a temperamental trait called “gregariousness” (Lansade et al., 2008); this latter trait might be analogous to underlying sociality as putatively expressed in vocalisation responses to OF and NO tests in our dairy cattle. Correlating the rate of dairy cattle vocalisations during OF and NO tests with existing measures of sociality such as social reinstatement responses to conspecifics during a social runway test or the distance to the nearest neighbours in the home-pen (Gibbons et al., 2010) would provide a further rigorous test of this hypothesis.

As well as underlying sociality, we have suggested the existence in dairy cattle of another trait underlying locomotor responses to OF and NO tests (Van Reenen et al., 2004, 2005). At the age of 7 months, locomotion of Cohort 1 animals during OF and NO tests was positively correlated with the time spent struggling during restraint (Van Reenen et al., 2004). Since this latter behaviour is generally believed to be an important marker of coping style in pigs (e.g., Hessing et al., 1994; Erhard et al., 1999; Bolhuis and Schouten, 2002; Geverink et al., 2002), coping style was proposed as a candidate trait in this respect (Van Reenen et al., 2004, 2005). High levels of OF locomotion and struggling during restraint would then reflect the propensity of the individual to adopt an active rather than a passive coping style, respectively (Koolhaas et al., 1999). A possible link at trait level between OF locomotion and behavioural resistance or agitation during challenge in bovines is supported by the present findings that individual differences in struggling during restraint at 7 months of age reliably predicted individual differences in OF locomotion at 22 months in Cohort 1 heifers. Such a relationship is also supported by studies in beef cattle where the level of OF ambulation was positively associated with flight speed from a crush and the time taken by a human handler to restrain individual animals in the corner of a test arena other than the OF (Kilgour et al., 2006; Müller and Keyserlingk, 2006). It is important to note that the explanation of locomotion in terms of coping style assumes that differences in locomotion are causally triggered by an emotional state of the animal such as fear or anxiety (Van Reenen et al., 2005; Koolhaas et al., 2007; Coppens et al., 2010). Thus, given the same emotional state (e.g., of fear), two animals may resort to different behavioural responses depending on their coping style: high locomotion (e.g., fear-induced activity or escape) in active copers and low locomotion (e.g., fear-induced immobility) in the case of a passive one (see Van Reenen et al., 2005). Alternatively, the locomotion of dairy cattle during OF and NO tests may be controlled independently of an emotional state, for example, by a “general activity” trait

(e.g., Ramos and Mormède, 1998; Andersen et al., 2000b) or a tendency to explore (e.g., Munksgaard and Jensen, 1996). In studies of cows (Kilgour, 1975) and heifers (Boissy and Bouissou, 1995) significant, but mostly moderate, correlations were found between locomotion and vocalisations in the OF, which led to the suggestion that underlying sociality may also be implicated in the regulation of OF locomotion in bovines (Boissy and Bouissou, 1995). Overall, a multifactorial regulation of OF locomotion (and possibly other measures of behavioural activity) in (dairy) cattle seems to be most consistent with the available data, and may also explain the significant correlation in the current study between the time spent struggling during the restraint test at 7 months of age and the number of vocalisations during the OF test at 22 months of age in Cohort 1 heifers (Table 6.4). The use of more sophisticated behavioural measures of coping style in future work with (dairy) cattle, e.g. those related to behavioural flexibility and the development of routines (e.g., Bolhuis et al., 2004; Coppens et al., 2010), may further elucidate the extent to which OF locomotion is mediated by coping style or other predispositions.

Underlying fearfulness was proposed as a third characteristic that might be involved in the regulation of dairy cattle's responses to OF and NO tests (Van Reenen et al., 2004, 2005). More specifically, we postulated that underlying fearfulness is reflected in an aggregate measure of behavioural and adrenocortical responsiveness to novelty, represented by the first principal component obtained after PCA (Van Reenen et al., 2005). Fearful calves were assumed to exhibit avoidance of the novel object (i.e., a high latency to first contact and a short time spent in contact with it) as well as high cortisol responses to OF and NO tests, whereas non-fearful ones were expected to show the opposite response pattern. This notion was supported by recent findings that in a combined OF and NO test the anxiolytic agent brotizolam increased the time calves spent in contact with the novel object and accelerated the post-test decrease in plasma cortisol concentrations (Van Reenen et al., 2009). Importantly, brotizolam did not affect vocalisation or locomotion during the OF phase of the test nor vocalisation following the introduction of the novel object; this is consistent with the view that these measures reflect characteristics other than fearfulness. The overall pattern of correlations obtained in the present experiment reveals that putatively fearful 6-month-old calves subsequently showed relatively high cortisol responses to OF and NO tests and, to some extent, heightened cardiac responses to confinement in the start box at 29 months of age. This finding closely agrees with a study in rats by Cavigelli and McClintock (2003) who found that high avoidance of novelty during infancy was associated with increased

corticosterone responses to brief psychological stressors (i.e. exposure to a novel environment or restraint) during adulthood. Correspondingly, these authors made the distinction between “neophobic”, or fearful, and “neophyllic”, or non-fearful individuals, and argued that these response styles in rats are analogous to “inhibited” and “uninhibited” children (Kagan et al., 1988, 1998), or “fearful” and “relaxed” monkeys (Suomi, 1991; Byrne and Suomi, 2002). Here, we reason that a similar classisification may apply to dairy cows with differential behavioural and adrenocortical response patterns to NO and OF tests.

In our tests, the main correlates of underlying fearfulness in dairy calves seem to consist of the behavioural response to the novel object (contact latency, duration of contact) and the cortisol responses to OF and NO tests. However, in 6-month-old calves locomotion during the NO test was also correlated with the aggregate measure of fearfulness (i.e., the first principal component) in that high avoidance of the novel object and high cortisol responses were associated with low levels of locomotion, and vice versa (Van Reenen et al., 2005). In agreement with this proposed relationship, locomotion in the NO test at 6 months of age was negatively correlated with the cortisol response to the OF at 29 months in the present study. It seems that locomotion during the NO test cannot be exclusively attributed to a single underlying trait, this supposition is also indicated by the correlation between locomotion during the NO test at 29 months and vocalisation during the NO test at 6 months of age (Table 6.8). In this context it is interesting to note that the brotizolam-induced increase in locomotion was dose-dependent and most noticeable within 1.5 m of the novel object (Van Reenen et al., 2009) thus suggesting that in a NO test locomotion close to the object and locomotion away from it may be controlled by different underlying factors.

Notably, in comparison to the rearing period adult cows paid much less attention to the novel object in the NO test. Moreover, at 29 months of age there were no relationships between interaction with the novel object and adrenocortical responses to OF and NO tests. One explanation may be that the novel object no longer constituted a sufficiently novel or challenging stimulus for adult cows and therefore no longer elicited individual differences in fear responses. This may also explain the absence of inter-test consistency of individual differences in the behavioural response of cows to novel stimuli presented in the home environment (Herskin et al., 2004). It could be argued that repeated exposure over time to a range of experiences may have progressively habituated the Cohort 2 heifers to our novel object and thus exerted a strong reduction in behavioural and adrenocortical stress responses (e.g., Levine et al., 1989). However,

there were no differences between 29-month-old cows and 6-month-old calves in the average adrenocortical sensitivity (ratio between cortisol and ACTH after CRH challenge) or in the average cortisol response to the NO test. This suggests that calves and adults perceived the NO test to be just as stressful, and that any possible habituation would have been stimulus specific, i.e. restricted to the novel object.

Developmental changes may also have influenced repeated test responses in our animals. Murphey et al. (1981) confronted different age groups of cattle with a novel stimulus (i.e., an unfamiliar person lying on the ground), and found that yearling heifers showed more investigative behaviour than mature cows. Similarly, adolescent rats and mice display more complex and varied behavioural patterns than adults and are willing to take more risks during behavioural tests of anxiety (Macri et al., 2002; Ray and Hansen, 2005). Perhaps the more pronounced individual differences in the responses of 6-month-old calves to the novel object simply reflected a greater variability of adolescent behaviour. Such a maturational effect might also explain the notable reductions with age in vocalisation, particularly during the OF test in Cohort 1 and the NO test in Cohort 2 animals. Developmental work with rats and mice has suggested both an increase (e.g., Macri et al., 2002) and a decrease (e.g., Lynn and Brown, 2010) with age in anxiety apparent in various test paradigms. Therefore the possibility remains that the markedly reduced interaction with the novel object in Cohort 2 heifers during first lactation reflected increased fearfulness rather than stimulus specific habituation.

A moderately positive association between the cortisol response to CRH challenge (area under the curve) and the cortisol response to the NO test at 29 months of age provides some indication that fearfulness may affect HPA axis function in dairy cows. Possibly, over time, fearful animals may have more frequently exhibited enhanced adrenocortical responses to environmental stressors than non-fearful animals, which eventually increased their adrenal sensitivity. Exposure to chronic environmental stress, however, may lead to desensitization of the HPA axis in bovines (Mormède et al., 2007). This might explain the apparent disagreement between the present findings and a recent study in beef cattle showing reduced cortisol output after CRH challenge in “temperamental” animals compared to “calm” ones (Curley et al., 2008). The latter authors argued that their “temperamental” cattle might be in a state of chronic stress.

In Cohort 1 heifers, interaction with a novel object during a NO test at 7 months was unrelated to inhibition of milk ejection or to stepping and kicking behaviours during first machine milking. Thus, on the assumption that interaction with a novel object reflects underlying fearfulness, the present study would suggest that fearfulness does not affect

the responsiveness of dairy cows to milking. Fear of humans, however, has been implicated in reduced milk letdown of dairy animals in a number of studies, including our own with Cohort 1 heifers (Lyons, 1989; Van Reenen et al., 2002). A possible explanation is that the response of dairy cows to frightening stimuli is situation or context specific, e.g. a cow that is fearful of a novel object may not be fearful of a human and vice versa. Dissociation between (fear) responses to humans and to inanimate objects was observed in bovines (Hemsworth et al., 1996; Gibbons et al., 2009a), pigs (Janczak et al., 2003a), and horses (Visser et al., 2003), and is consistent with the concept of "domain-specificity" of personality (Wilson, 1998).

Intuitively, we might assume that stepping and kicking by cows in the milking parlour reflect the propensity to actively resist a challenge and would therefore be positively associated with putative measures of active coping such as struggling during restraint or OF locomotion. However, in Cohort 1 heifers there were no relationships between parlour behaviour and any other measure recorded during rearing or gestation. Similarly, OF locomotion or movement during isolation in a novel box were not related to behaviours during milking in cows (Kilgour, 1975) or sheep (Murray et al., 2009), respectively. Again, an element of "domain-specificity" of response mode might be involved here. An intriguing experiment by Schrader (2002) even suggested that high levels of step and kick responses (in this case to tail fixation) of dairy cows might be associated with enhanced behavioural flexibility, which is believed to be characteristic of animals with a passive coping style (Bolhuis et al., 2004; Coppens et al., 2010). Alternatively, stepping and kicking behaviour during milking may be related more to the aversion of teat cup attachment than to the emotional response to challenge (Uetake et al., 2004).

Inhibition of milk ejection was significantly associated with vocalisation in earlier OF and NO tests: heifers with high rates of vocalisation at 7 months of age exhibited better milk ejection on day 2 of lactation. Although single correlations should be evaluated with caution, we may tentatively suggest that underlying sociality might be implicated here. Thus, heifers with a high motivation for companionship may be more at ease in a situation such as milking, with close contact to conspecifics, and may be more receptive to stress-reducing effects of social support. A beneficial effect of conspecifics may possibly generalize to humans, including the milker, since calves vocalised much less during a response to human test than during OF and NO tests (Van Reenen et al., 2004), and the "Vocalisation" factor obtained after PCA in the present experiment had high and opposite loadings for vocalisations and the latency to first contact with the

human (Table 6.3). In Cohort 1 heifers, inhibition of milk ejection during early lactation milkings was inversely related to milk production (Van Reenen et al., 2002). The possible involvement of sociality in the regulation of milk letdown is therefore supported by the observation that OF vocalisations of dairy cows kept in loose housing for the first time were positively correlated to milk production at the beginning of lactation (Kovalcikova and Kovalcik, 1982/83), but more research is necessary to confirm this supposition. Studies in poultry already provided more substantial evidence that high social motivation may increase production efficiency in group-housed farm animals. Broiler chicks that negotiated a T-maze quickly to re-establish contact with their conspecifics had higher subsequent growth rates than their slower counterparts (Marin et al., 1999, 2003).

CONCLUSION

The present experiment demonstrated long-term consistency of individual differences in behavioural and adrenocortical responses of dairy cattle to acute stressors. This finding supports the idea that stress responsiveness in dairy cows is mediated by developmentally stable underlying traits. The patterns of correlations reported in this study agree with the existence of multiple independent dimensions of reactivity. These are likely related to vocalisation, locomotion and correlated adrenocortical and behavioural responses to novelty, and may reflect underlying sociality, activity (coping style) and fearfulness, respectively. Inhibition of milk ejection and stepping and kicking behaviours during first machine milking were not related to earlier struggling during restraint, locomotor responses to OF and NO tests, or the behavioural interaction with a novel object. However, the relationship between ease of milk letdown and the rate of vocalisations during OF and NO tests suggests that underlying sociality might be implicated in the inhibition of milk ejection, but more research is necessary to confirm this.

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CHAPTER 7

General discussion

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INTRODUCTION

The main aim of the work described in the present thesis was to examine the consistency of individual differences in behavioural and physiological responses to acute stressors in dairy cattle in a longitudinal fashion, i.e. both during rearing and in adulthood (chapters 2, 3 and 6). Information about the variation in the reactivity of dairy cattle to a "real-life" challenge was provided by a study of heifers' responses to first-time machine milking (chapter 5). A pharmacological validation experiment helped to interpret the response patterns that calves exhibited in behavioural tests designed to induce stress (chapter 4). The long-term longitudinal study (chapter 6) enabled clarification of the relationship between responses of heifers to first machine milking and their earlier responses to behavioural tests. In this general discussion, I will consider the results in a wider context, and also specifically focus on (i) the multidimensional nature of response patterns to stress, (ii) the significance of individual differences in stress responsiveness in (farm) animals, and (iii) some practical implications of the findings of this thesis.

MULTIDIMENSIONAL RESPONSE PATTERN TO STRESS

The dominant picture provided by the results of this study is that the responsiveness of dairy cattle (both cows and calves) to challenge is mediated by multiple traits, i.e., is multidimensional. Applying principal component analysis (PCA) to behavioural and physiological response measures always yielded at least two components (dimensions) without cross-loading of variables, even when it was widely assumed that those measures could be related to the same trait (see chapters 2, 3 and 5). This seems to contrast with prevailing concepts of individual differences which emphasized the existence of major unifying dimensions such as, for example, fearfulness (Boissy, 1995, Jones, 1996), temperament (Clarke and Boinski, 1995), behavioural syndrome (Sih et al., 2004a, b) or coping style (Koolhaas et al., 1999, see Korte et al., 2005 for a related characteristic defining the unidimensional distinction between "hawk" and "dove" type personalities in animals and men). The basic premise of these concepts is that animals exhibit consistent individual differences in behavioural and physiological responses across a wide range of different environmental challenges. However, from many reports in the literature, covering many species, it appears that multidimensional response patterns to stressors seem to be the rule rather than the exception. For example, multiple independent characteristics were obtained after correlational analyses, including PCA, of behavioural and physiological measures of

response to challenge in sunfish (Coleman and Wilson, 1998), geese (Kralj-Fiser et al., 2006), quail (Mignon-Grasteau et al., 2003; Miller et al., 2006), deer (Pollard et al., 1994; Bergvall et al., 2011), laboratory rodents (Kanari et al., 2005; Ibanez et al., 2007, 2009), dogs (Svartberg, 2005), pigs (Forkman et al., 1995; Spoolder et al., 1996; Mendl et al., 1998; D'Eath and Burn, 2002; Janczak et al., 2003a, b; Van Erp – van der Kooij et al., 2002; Brown et al., 2009), and cattle (Piovezan et al., 1998; Kilgour et al., 2006; Petherick et al., 2009a). Clearly, there is a need for alternative interpretations of individual differences that go beyond univariate classifications.

Domain-specificity of response

Using the temperamental trait "boldness" (or the "shy-bold continuum") as an example, Wilson and colleagues (Wilson et al., 1994; Wilson, 1998) argued that, depending on the ecological circumstances, it might be favourable for an individual to facultatively express either a shy or a bold reaction pattern in the face of challenge (e.g., novelty, a predator, a conspecific, etc.). This was described as "domain specificity" or "phenotypic plasticity" of response, as opposed to "domain generality" or "phenotypic rigidity" (see also Réale et al., 2000; Sih et al., 2004a, b). A similar context dependency of response was proposed for characteristics like fearfulness, anxiety, or emotionality (Archer, 1979; Ramos and Mormède, 1998). This might explain, for example, why the same animal is fearful of humans, but non-fearful of novel objects (e.g., Visser et al., 2003; Janczak et al., 2003a; Gibbons et al., 2009a), or aggressive and agitated towards conspecifics, but non-aggressive and non-agitated during manual restraint or handling (e.g., Mendl et al., 1998; Réale et al., 2000; D'Eath and Burn, 2002), etc. The current findings in dairy cattle, however, show that different dimensions of responsiveness not so much differed with regard to the context (i.e., the test) in which behavioural and/or physiological measures were obtained, but that each dimension largely reflected the same measure recorded in different tests, i.e., locomotion, vocalisation or correlated behavioural and adrenocortical responses to novelty (see chapters 2, 3 and 6). Explaining the present results only in terms of context specificity of response would, therefore, require a rather complex line of argument because it would not only mean that the same underlying trait (for example, fearfulness) is differentially expressed (i.e., by vocalisation, by locomotion, or by correlated behavioural and adrenocortical responses to novelty) depending on the context (i.e., different tests, or different aspects of the same test such as novelty or social isolation), but also that different forms of context dependent

fear responding are simultaneously exhibited in the same animal (see chapter 2). Thus, it is worthwhile to consider other frameworks too.

Qualitative and quantitative dimensions of responsiveness to challenge

Another explanation more closely considers the nature of two main concepts of individual differences: coping style and underlying fearfulness. The concept of coping style assumes that individuals may show alternative types of response patterns to the same challenge, e.g., either a passive or an active type of reaction (Koolhaas et al., 1997, 1999). Importantly, passive coping by no means indicates that animals are "passive" in the sense that they do not respond. On the contrary, a passive coping strategy is thought to include, for example, enhanced parasympathetic activity and, under some circumstances, a high reactivity of the hypothalamo-pituitary-adrenocortical (HPA) axis (Koolhaas et al., 1999; Korte et al., 2005), and it was therefore suggested to rename the word "passive" into "reactive" (Koolhaas et al., 1997). The concept of fearfulness, in contrast, makes a distinction between, on the one hand, fearful animals that are highly emotionally aroused by a challenging situation and, hence, exhibit activation of neuroendocrine systems involved in stress responsiveness (such as the HPA axis and the sympatho-adrenomedullary system) and, on the other, non-fearful or "relaxed" animals who do not perceive that same situation as stressful or alarming and, therefore, do not show any enhanced, or at least show less elevated, biological responses (Boissy, 1995; Jones, 1996). Thus, coping style seems to reflect the type of response an animal makes (i.e., *how* an animal reacts), and fearfulness indicates the level of responsiveness to challenge (i.e., *how strongly* an individual reacts). From a conceptual point of view, coping style would then represent the *qualitative* dimension of the stress response, and fearfulness the *quantitative* one. In the present thesis, this two-dimensional or "two-tier" model (see Koolhaas et al., 2007; Coppens et al., 2010) was proposed to explain the multivariate response pattern of calves to open field (OF) and novel object (NO) tests (chapter 3). Correlated behavioural and adrenocortical responses of calves to novelty were suggested to reflect underlying fearfulness, while OF locomotion was thought to be mediated by a type of coping style. The assumptions of this model were (i) that in the NO test paradigm all fearful calves adhered to the same behavioural strategy of response (i.e., they had long latencies to first contact with the NO, and spent short times in contact with it), (ii) that the level of responsiveness (i.e., the level of avoidance of the NO, and the strength and amplitude of plasma cortisol responses), was higher in fearful calves than in non-fearful ones, and (iii) that during the

OF test, both fearful and non-fearful calves were equally likely to consistently exhibit either high or low levels of locomotion, possibly under the influence of coping style. These assumptions would predict that the administration of a fear-reducing pharmacological agent to calves prior to OF and NO tests would decrease the strength of the fear response (i.e, decrease avoidance of the NO and, hence, increase their interaction with it and decrease the magnitude of the cortisol response), but would not affect OF locomotion. These predictions were largely confirmed in the pharmacological validation study (chapter 4), thereby lending important experimental support to the model. A crucial implication of this model would be that the nature of the experimental paradigm (e.g., the type of behavioural test) that is used to assess individual differences in responsiveness to challenge determines the extent to which fearful animals will exhibit similar or, under the influence of coping style, differential response patterns to the same challenge. If we accept the argument that some paradigms, or some behavioural or physiological measures recorded within the same paradigm, primarily reflect the level of responsiveness (e.g., fearfulness), and others mainly indicate the qualitative type of response (e.g., coping style), then *by definition* the resulting overall response pattern should be multidimensional if both types of paradigms are used, or both types of measures are recorded, in the same experiment (see Ramos and Mormède, 1998). A more conceptual version of the two-dimensional model of responsiveness previously presented in chapter 3 is shown in Figure 7.1. The horizontal axis represents the quantitative dimension of the response pattern to challenge, which encompasses the strength and the amplitude of the response. These may vary between high (left-hand side) and low (right-hand side). The horizontal axis represents the qualitative dimension of the response, with animals on both extremes of the distribution differing in, for example, the type of coping style. (e.g., reactive versus proactive).

I suggest that a closer inspection of previous papers, in particular those on the species most frequently used to examine coping style (i.e., laboratory rodents and pigs), provides further support for multidimensionality due to the dissociation between “level” and “type” of response. For example, Koolhaas et al. (2001) reported that the attack latency of wild type male rats in a resident intruder test (an index of coping style in rats and mice, see Benus et al., 1991) was unrelated to the time spent in the open arm of the elevated plus maze (an index of fear and anxiety, see Pellow et al., 1985). Correspondingly, Sgoifo et al., (1996) found that the attack latency of rats was significantly associated with plasma catecholamine concentrations after social (defeat experience) or non-social (exposure to a shock prod in the home pen) stress (reflecting

the level of activation of the sympathetic nervous system, a neuroendocrine index of coping style, see Koolhaas et al., 1999; Korte et al., 2005), but unrelated to stress-induced plasma corticosterone (which has been suggested to reflect the level of fear or anxiety, e.g., Landgraf and Wigger, 2003; Marquez et al., 2005). Similarly, in pigs, putative measures of coping style, such as the behavioural response to a backtest or the level of aggression during social confrontations with conspecifics (see Hessing et al., 1993; Ruis et al., 2000; Bolhuis et al., 2004, 2005), were mostly unrelated to putative measures of fearfulness or anxiety, such as the latency to approach a novel object or a human (e.g., Jensen, 1994, Jensen et al., 1995a; Forkman et al., 1995; Spoolder et al., 1996; Van Erp – van der Kooij et al., 2002; Janczak et al., 2003b; Brown et al., 2009).

On the assumption that a two-dimensional model of responsiveness based on the interaction between the level of responsiveness and the qualitative type of response is valid, one could surmise that an emotional state of fear – which would be most apparent in animals situated on the right-hand side of the two-dimensional distribution presented in Figure 7.1 – is a prerequisite for the expression of individual differences in coping style. Studies of mice (Sluyter et al., 1996) and piglets (Erhard et al., 1999) favoured this idea by suggesting that animals only differed in putative behavioural measures of coping style (i.e., defensive burying or tonic immobility in mice and piglets, respectively) when they experienced the test situation as stressful or aversive (i.e., when mice were tested with fresh bedding, or when piglets exhibited behavioural signs of emotional distress in response to handling). Steimer et al. (1997) and Koolhaas et al. (2007), on the other hand, proposed two-tier models of responsiveness, similar to the model in Figure 7.1, where differences in coping style might also be expressed under low fear circumstances, for example in differential levels of impulsiveness. In the current thesis it was suggested that in non-fearful animals OF locomotion might be controlled by characteristics other than coping style, such as the tendency to explore (chapters 3), but this needs further research. It would, however, also be perfectly conceivable that a coping style trait in dairy cattle mediates both the need to explore in non-fearful animals as well as the expression of locomotion (i.e., either fear-induced immobility or fear-induced activity) in fearful ones.

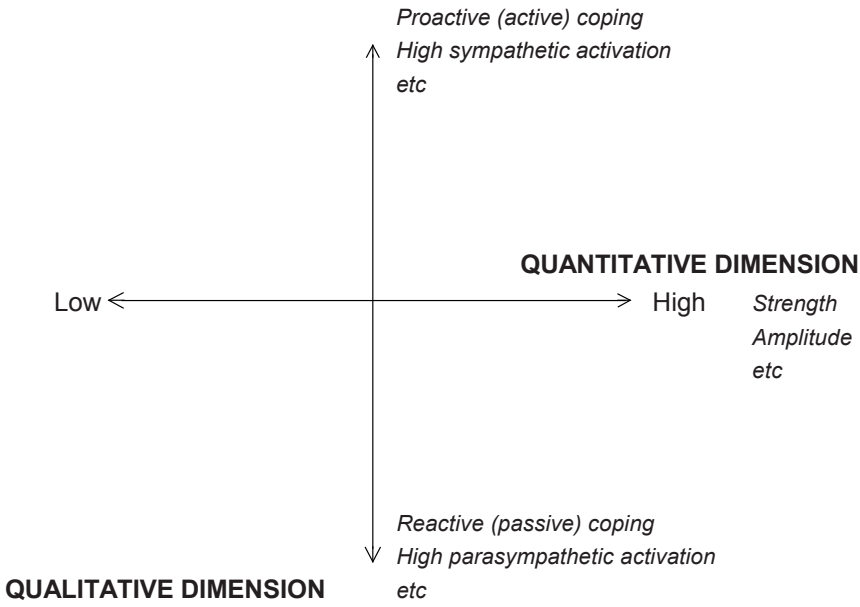

Figure 7. 1 Model of responsiveness of animals to challenge, based on two independent (orthogonal) dimensions: a quantitative dimension (horizontal axis) representing features like the strength and amplitude of the response, and a qualitative dimension (vertical axis) reflecting the qualitative type of reaction. Animals can be distributed in the two-dimensional space according to their behavioural and physiological response pattern to various challenges. Animals on the far left-hand side of the quantitative dimension experience low emotional arousal, and have baseline states of the neuroendocrine systems involved in the stress response, whereas animals on the right-hand side of the dimension experience high levels of fear or emotional distress, and exhibit increased activity of neuroendocrine response systems in reaction to the same challenge. Animals situated on the extremes of the qualitative dimension exhibit differences in, for example, type of coping style (e.g., reactive versus proactive) or nervous system balance (e.g., high parasympathetic versus high sympathetic activity). Animals are assumed to exhibit individual differences in the level of responsiveness, and/or in the qualitative type of response, depending on the nature of the (experimental) challenge or paradigm they are subjected to.

Sociality

It is important to note that neither context specificity of response, nor the recognition of qualitative and quantitative dimensions of fear responses to stressors, seemed sufficient to fully explain the present response patterns observed in dairy cattle. Underlying sociality (or sociability), i.e. the motivation to remain close to conspecifics (Erhard and Schouten, 2001; Sibbald et al., 2006; Gibbons et al., 2010) was proposed as a separate trait underpinning the responsiveness of calves and cows to the challenges examined here (chapters 2, 3 and 6). This trait is not only related to social separation distress but also to social bonding and the capacity to be comforted by peers, which are regulated by distinct reward areas in the brain (e.g., Massen et al., 2010). This explains why putative behavioural measures of underlying sociality in cattle, (e.g. vocalisations) recorded during social isolation or separation, were positively correlated with the duration of non-aggressive social interactions (including sniffing and licking) with conspecifics (Boissy and Bouissou, 1995; Boissy and Le Neindre, 1997). In the present thesis, it was hypothesized that the rate of OF vocalisation reflects sociality in dairy cattle (chapters 2, 3 and 6). This behavioural measure was largely uncorrelated with other behavioural and physiological measures. Therefore, the two-dimensional model presented in Figure 7.1 should be extended with at least one additional dimension, representing underlying sociality, perpendicular to the other two axes (see also chapter 3). For the time being, I assume that underlying sociality behaved like a quantitative trait, i.e., that the number of vocalisations reflected the strength of the response, with high and low levels of vocalisation referring to high and low levels of sociality, respectively. Theoretically, however, qualitative dimensions of sociality cannot be completely ruled out, but remain speculative.

Genetic studies

The concept of multidimensionality of response patterns to challenge is also supported by (molecular) genetic studies. Gutierrez-Gil et al. (2008), for example, searched for genomic regions (i.e., quantitative trait loci or QTLs) influencing behavioural traits pertaining to cattles' response patterns exhibited during exposure to social separation and flight distance tests. Notably, QTLs associated with traits assessed in different tests did not overlap, suggesting that different aspects of cattle "temperament" or "fearfulness" are controlled by different underlying genetic factors. Earlier work in quail (Mills and Faure, 1991; Faure and Mills, 1998) has revealed that genetic lines can be

created showing either high or low levels of social reinstatement behaviour (a measure of sociality), or long or short durations of tonic immobility (a commonly used measure of fearfulness). This suggests that sociality and fearfulness are indeed independent traits, and supports the current hypopthesis that these traits are also independently expressed in dairy cattle (see chapters 2, 3 and 6). Conversely, extensive studies in genetic lines of a wild bird species, the great tit (*Parus major*), indicated that behavioural profiles may consist of a range of genetically correlated traits, including risk taking behaviour, aggression, exploration, and interaction with a novel object, which would argue in favour of the existence of common sets of genes that exert an influence on multiple characteristics that shape the way these birds cope with environmental challenge (Van Oers et al., 2004; Carere et al., 2005; Groothuis and Carere, 2005). Under natural circumstances, however, selection pressure may also act on multiple independent traits simultaneously, producing behavioural syndromes comprised of phenotypically rather than genetically correlated traits (e.g., Sih et al., 2004a, b; Réale et al., 2007; Wolf and Weissing, 2010). Moreover, behavioural and physiological differences between genetic lines should be treated with care even under controlled experimental conditions. Such differences may be the result of fortuitous or accidental co-selection of genetically unrelated traits, and, therefore, systematic breeding experiments are required to identify true genetic links (see Castanon and Mormède, 1994; Mormède et al., 1994; Ramos and Mormède, 1998; Groothuis and Carere, 2005). The use of high-low sampling from a phenotypic distribution, as a tool to create groups with divergent response profiles (e.g., Ruis et al., 2000; Geverink et al., 2004), may introduce similar risks, in particular when different independent traits are not equally distributed among the population of interest.

Multidimensionality of temperament across species

The present suggestion that responsiveness to challenge in dairy cattle is mediated by multiple independent traits, including fearfulness, sociality and coping style, is supported by a considerable body of literature. Multiple traits (e.g., the “Big Five”) are also assumed to constitute human personality (e.g., Zuckerman, 1991; Funder, 2001), and a multidimensional approach is increasingly advocated in the context of research into animal personality, behavioural syndromes or coping style (e.g., Erhard and Schouten, 2001; Gosling, 2001; Visser, 2002; Sih et al., 2004a, b; Koolhaas et al., 2007), even to the point where it was proposed that animal temperament actually consists of five categories of traits (some of which may or may not be genetically linked), including “aggressiveness”, “avoidance of novelty”, “willingness to take risks”, “exploration”, and

"sociability" (Réale et al., 2007). I argue that the application of a multidimensional model of responsiveness, based on the fundamental distinction between qualitative and quantitative dimensions of behavioural and physiological responses of animals to challenge, could be one way of reconciling the concept of fearfulness with that of coping style. For one thing, this model may help to explain multidimensional response patterns without questioning the validity of either concept, as has happened previously (e.g., in the case of coping style in farm animals, particularly pigs, see Jensen, 1995; Jensen et al., 1995b; Spoolder et al., 1996; Blokhuis et al., 2001). At the same time, in contrast with suggestions made by some authors (e.g., Jones, 1996; Landgraf and Wigger, 2003; Carere et al., 2005), this model would question the idea that coping style and fearfulness are similar or the same traits.

SIGNIFICANCE OF INDIVIDUAL DIFFERENCES

Individual differences as a consequence of natural selection

It is becoming increasingly clear that animals in the wild are exposed to selection pressures that facilitate the development and maintenance of consistent individual differences in behaviour within the same population, and that trade-offs play a key role in this respect (see Sih et al., 2004a, b; Stamp, 2007; Wolf et al., 2007; Wolf and Weissing, 2010). For example, a trade-off may exist between current and future growth and reproduction, and, hence, some animals may adopt a behavioural strategy that maximizes growth and reproduction in the short term, whereas others may consistently behave in such a way that growth and reproduction are safeguarded in the long run. The former category of animals may exhibit "bold" and aggressive personality traits, whereas "shy" and more cautious personality traits may prevail in the latter category (e.g., "risk takers" versus "risk avoiders", see Wilson, 1994). Within each "life history strategy", the animal strikes a balance between benefits and costs, i.e., "bold" animals have a fast growth and a high reproductive rate at the cost of higher risks of injury and mortality due to predation, and "shy" animals exhibit slower growth and lower reproductive rates, but at the same time their risk of injury and mortality is also lower (see also Korte et al., 2005). Consequently, different personality types may ultimately obtain equal overall fitness in terms of gene preservation. Similarly, there may be trade-offs between adaptation to different social and environmental conditions in migratory species. For example, in wild house mice aggressive animals are the most successful under stable territorial conditions, whereas non-aggressive ones thrive during emigrations and the

establishment of new territories (Van Oortmerssen and Bussen, 1989): these types are believed to represent animals with a proactive (active) or a reactive (passive) coping strategy, respectively (Benus et al., 1991; Koolhaas et al., 1997, 2001). Thus, under natural circumstances, when animals of the same population are faced with the same challenging situation, consistent individual behavioural differences may be linked to profound differences in health and other life outcomes such as reproductive success (see Dingemanse and Réale, 2005; Korte et al., 2005; Réale et al., 2007). Behavioural differences of this sort have therefore been referred to as "adaptive personality differences" (e.g., Dingemanse and Wolf, 2010; Wolf and Weissing, 2010).

Biological basis of individual differences and temperament

Comprehensive animal models are beginning to unravel the neurobiological, immunological and genetic factors and mechanisms underlying the relationship between personality and health, and noticeable relations between behavioural characteristics and aspects of immunocompetence and disesase susceptibility have been reported (e.g., Landgraf and Wigger, 2003; Cavigelli, 2005; Korte et al., 2005; Kavelaars and Heijnen, 2006; Koolhaas et al., 2006; Koolhaas, 2008; Salome et al., 2008). For instance, individual differences in behavioural measures of coping style in mice and rats, such as the attack latency in a resident intruder test, are associated with differences in the propensity to develop stress-related pathologies (e.g., hypertension and atherosclerosis), and in the susceptibility to autoimmune disease (Koolhaas, 1994; Kavelaars et al., 1999). Another consistent behavioural characteristic in rats, locomotor activity in an open field, correlated with the propagation of injected tumor cells and with the progression of experimentally induced arthritis (Sajti et al., 2004a, b). Divergent genetic selection in rats for a neurobiological characteristic, i.e., sensitivity to the dopaminergic agonist apomorphine, resulted in lines that differ in a wide range of biological variables, including behavioural and physiological responses to novel and social challenges, and immunological reaction patterns to inflammatory (autoimmune) and infectious diseases (Cools et al., 1990, 1993; Kavelaars et al., 1997, Teunis et al., 2004). These studies provided confirmation of the now widely accepted notion that intricate and reciprocal relations exist between the central nervous, endocrine, and immune systems (see Ader et al., 1995; Glaser and Kiecolt-Glaser, 2006). Personality traits may then be viewed as fundamental moderating or intervening variables that, in a hierarchical sense, operate at a high level in the body (i.e., in the brain), and affect both the intensities and the types of

a wide range of biological responses the individual mounts during challenge (see Boissy, 1995; McEwen, 2001; Korte et al., 2005).

Individual differences in domestic animals

Comparative research shows that similar (co)variations in behavioural and physiological response patterns to challenge exist in a wide range of vertebrate species, from fish to mammals, including humans (e.g., Wilson, 1994; Gosling, 2001; Sih et al., 2004a, b; Øverli et al., 2007; Mehta and Gosling, 2008). This suggests that similar "adaptive personality" traits have been conserved during evolution across species, involving common biological (e.g., neural and neuroendocrine) substrates, and, possibly, homologous genes (Flint et al., 1995; Mormède et al., 2002; Øverli et al., 2007). In comparison with their wild ancestors, domestication has undoubtedly altered the physical appearance and the behaviour of farm animals in many ways. There is, however, reasonable consensus that most of these changes are quantitative rather than qualitative in nature, or, in genetic terms, that during domestication no genes have disappeared from the gene pool (Price, 1999; Jensen, 2001; Mignon-Grasteau et al., 2005). With regard to behavioural and physiological responses to challenge, this means that it is the amplitude and vigour of response that has mainly changed (i.e., mostly decreased in the course of domestication) rather than the variability and diversity in response repertoire. In other words, there is reason to assume that even in the absence of ancient selection pressures that originally shaped the emergence of "adaptive personality" differences in animals and man (see above), modern farm animals still harbour a considerable potential to express differential behavioural and physiological response patterns to challenge, and, correspondingly, experience different fitness (e.g., health) consequences under divergent environmental conditions.

Numerous examples in the scientific literature substantiate the above assumption. Studies in pigs demonstrated that (early) differences in the behavioural response to the backtest (a putative measure of coping style) or to other behavioural tests intended to provoke individual temperamental differences, were associated with differences in, for example, responses of the immune system to experimental antigenic challenges (Hessing et al., 1995; Bolhuis et al., 2003), growth, lean meat percentage and meat quality (Hessing et al., 1994; Van Erp – van der Kooij et al., 2000, Brown et al., 2007), maternal behaviour and reproductive success (Spinka et al., 2000; Thodberg et al., 2002a, b; Janczak et al., 2003a), and the performance of abnormal stereotypic behaviours (Geverink et al., 2003). Likewise, variation in struggling during restraint in a

chute (crush), or in flight speed (i.e., the time taken for an individual animal to cover a set distance when it is released from a chute or weighting crate), recorded in beef cattle prior to the fattening or service period, covaried with variation in later measures of growth and meat quality (Voisinet et al., 1997a, b; Petherick et al., 2002, 2009b; King et al., 2006), immune function (Fell et al., 1999; Oliphint et al., 2006), and pregnancy rate (Cooke et al., 2009). Behavioural temperament of Limousin heifers assessed during standard encounters with a human handler was genetically correlated with maternal behaviour (i.e., licking the new born calf) and fertility and calving rate (Phocas et al., 2006). In poultry, differences in a range of neuroendocrine and behavioural response patterns to brief experimental challenges were related to (subsequent) differences in growth (Marin et al., 1999, 2003), egg production (Uitdehaag et al., 2008a), and the propensity to engage in (potentially harmful) feather pecking behaviours directed to conspecifics (Van Hierden et al., 2002; Rodenburg et al., 2004; De Haas et al., 2010).

Individual differences in dairy cattle

So far, in comparison with other farm animal species, less research has been devoted in dairy cattle to the consequences of individual differences in stress responsiveness for the capacity of the animals to adapt to (challenging factors in) their actual living environment. Nevertheless, the available data seem to agree with the general picture emerging from studies in other species (see above). In Normandy cows (a French dairy breed), for example, behavioural and heart rate responses to unfamiliar test situations correlated with muscle characteristics, including temperature and pH, recorded at the slaughter house three weeks later (Bourguet et al., 2010). Schrader (2000) examined relationships in dairy cows between (i) behavioural reactions to brief experimental challenges, including “tail fixation”, (ii) measures of spontaneous activity in the home environment (cubicle house), calculated from 24-hour recordings of walking, standing and lying behaviour over a 4-day period, (iii) a measure of “regularity” of home pen behaviour, reflecting the day-to-day consistency of dairy cow activity in terms of the location in the barn (walking area, open yard, cubicles, feeding rack) where each animal was at each 5-min interval across the day, and (iv) an “agonistic index”, indicating for each individual cow the level of success during agonistic interactions. Significant and mutual correlations were found between the behavioural response (i.e., the number of steps and kicks) to tail fixation, the day-to day behavioural regularity, the average duration of lying bouts, and the agonistic index. It is tempting to speculate from this work that (consistency of) home pen activity and aggression in dairy cows are mediated by a

common underlying predisposition. Indeed, there is recent evidence that aspects of both home pen activity and aggressive behaviour in dairy cows possess trait-like qualities (Müller and Schrader, 2005; Gibbons et al., 2009b). The potential relevance of such a trait is further highlighted by results of Galindo et al. (2000), showing that an index of "displacements" (reflecting the extent to which an individual cow is displaced by other cows during agonistic interactions), and the time cows spent standing half in the cubicles, predicted the subsequent likelihood of animals becoming clinically lame. Hopster et al. (1998) selected primiparous heifers with either high ("high responders") or low ("low responders") plasma cortisol concentrations in reaction to an OF test, and subjected these animals to an experimental intra-mammary endotoxin challenge (mimicking a mild mastitis infection) during the subsequent lactation (i.e., one year after the initial selection). High and low responders significantly differed in the numbers of circulating lymphocytes between 10 and 21 hours post challenge. In the context of the present thesis, this is an especially intriguing finding because it would suggest that underlying fearfulness (putatively reflected in the cortisol response to an OF test, see chapters 3, 4 and 6) may affect aspects of the immune response against mastitis in dairy cows. Interestingly, there was only a transient difference between high and low responders in plasma cortisol (one hour post challenge), supporting the assumption that other (e.g., neural or neuroendocrine) factors contributed to the difference in lymphocyte numbers between the treatment groups (Hopster et al., 1998). This, in turn, would concur with the notion of central (i.e., seated in the brain) rather than peripheral coordination of the response to an immunological challenge (such as exposure to endotoxin), mediated by underlying personality characteristics such as fearfulness. The present thesis provides some evidence that underlying sociality may also be relevant to dairy cow's adaptation to regular husbandry conditions. The rate of OF and NO vocalisations at 7 months of age appeared to be correlated with inhibition of milk ejection during first machine milking more than one and a half years later (chapter 6). From a biological point of view, such a relationship would make sense on the assumption that vocalisation refers to a developmentally stable underlying trait that is capable of modifying the reactions of dairy cows to challenging situations at different ages and in different contexts.

Evaluating correlates of individual differences using a multidimensional approach

In the present thesis it is postulated that individual differences in stress responsiveness in dairy cows are mediated by multiple underlying traits (see above). A

truly multidimensional approach in the assessment and appreciation of the implications of individual differences in stress responsiveness, however, would have required that relationships were examined between, on the one hand, measures reflecting the capacity of the individual cow to adapt to its living environment (e.g., with regard to health or inhibition of milk ejection), and, on the other, (co)variations in multiple traits obtained with the use of experimental challenges (i.e., the behavioural tests). In terms of the model in Figure 7.1, it would then be the position of an animal in a multidimensional space that determines its adaptive capacity, rather than its position relative to an individual dimension or trait (such as fearfulness, coping style or underlying sociality). This comes close to the concept of a multivariate "fitness landscape", where "fitness" (e.g., survival, reproductive success, health, etc.) of animals in the wild is a function of multiple behaviours and their interaction (see Dingemanse and Réale, 2005, for an example with two behavioural traits "x" and "y", measured on each individual, e.g., "aggressiveness" and "risk taking behaviour", or "activity in the presence versus absence of predators", etc.). In the current thesis, with only 23 heifers available for studying the relationship between early reactivity to behavioural tests and later responsiveness to a "real life" challenge (see chapter 6), the number of animals was deemed insufficient to allow for such a multivariate approach in a statistically reliable way. Other studies, however, have already indicated that this could be a useful approach. Cavigelli et al. (2009), for example, distinguished two independent traits in rats exposed to novel stimuli related to behavioural inhibition and glucocorticoid production, respectively, and demonstrated that the lifespan of rats that exhibited both high behavioural inhibition and high glucocorticoid production was almost two months shorter than that of rats that showed either high behavioural inhibition alone, or high glucocorticoid production alone. Likewise, in humans, the combination of "depression", "hostility", and "anxiety" was a stronger predictor of coronary heart disease than any of these personality traits alone (Boyle et al., 2006; Mehta and Gosling, 2008). Based on extensive work with lines of rats selectively bred for differences in their behavioural performance in an active avoidance test, Steimer et al. (1997) suggested that the propensity to develop mental disorders may rely on the interaction between two independent dimensions, i.e. "emotional reactivity" (ranging between high and low, i.e., similar to fearfulness) and "coping style" (either "active" or "passive", and related to locomotor and rearing activity in a novel environment). Animals with a passive coping style and a high emotional reactivity (for a comparison, see lower right quadrant of Figure 7.1) would be susceptible to anxiety problems, whereas animals with an active coping style and a low emotional reactivity

(see upper left quadrant of Figure 7.1) would be prone to impulsiveness and a lack of behavioural inhibition (Steimer et al., 1997).

Temperament, fitness and welfare

In the context of animal ecology, the term “fitness” generally encompasses two main aspects, i.e., reproductive success and survival rate, both of which ultimately determine the ability of the individual to propagate its genes (e.g., Barker, 2009). Differences in temperamental or “adaptive personality” traits, in turn, are believed to exert an important influence on “fitness” (see Sih et al., 2004a, b; Dingemanse and Réale, 2005; Réale et al., 2007; Dingemanse and Wolf, 2010; Wolf and Weissing, 2010). In the context of the breeding and husbandry of domestic animals, the term “fitness” is used rather generically, and may either refer to an equivalent notion of “robustness”, or to aspects like fertility, disease resistance, health, and longevity (e.g., Calus, 2006; Van der Werf, 2007; Gibbons, 2009; Goddard, 2009; Ten Napel et al., 2009). Regardless of terminology, the present discussion argues that, similar to wild animals, the “fitness” (or “robustness”) of domesticated farm animals, including dairy cattle, is mediated by stable underlying temperamental traits. In the case of dairy cattle, I suggest that at least three traits may be relevant in this respect: fearfulness, sociality and activity (or coping style). Because reduced “fitness” (or “robustness”) may be associated with problems that involve elements of mental suffering (e.g., pain in the case of disease), variations in these temperamental traits may also have implications for animal welfare (see Duncan, 2005; Broom, 2007; Dawkins, 2008).

PRACTICAL IMPLICATIONS

Negative effects of selection for increased productivity

Although modern farm animals may not now be affected by most of the natural selection pressures that originally shaped the genotype of their wild ancestors, they are, however, continuously subjected to another major source of genetic change, resulting from selective breeding for production traits. Persistent artificial selection for production traits has tremendously increased production efficiency and production levels in all farm animal species over the last few decades (see Rauw et al., 1998; Sandøe et al., 1999; Oltenacu and Algers, 2005). At the same time, however, there is increasing evidence suggesting that intense selection for increased productivity has negatively affected the fitness of farm animals because of unfavourable genetic correlations between production

traits and measures of health, fertility and longevity (see Rauw et al., 1998; Sandøe et al., 1999; Oltenacu and Algers, 2005; Oltenacu and Broom, 2010).

The resource allocation theory is influential in explaining negative side-effects of selective breeding; this predicts that in farm animals, because of a disproportionate emphasis on biological processes related to production traits (e.g., growth or the production of milk), fewer resources (e.g., in terms of feed intake, body tissue, energy, etc.) are available for other important life functions such as reproduction or immune defence (Beilharz et al., 1993; Rauw, 2009). This may compromise the animal's adaptive capacities, and render it susceptible to disease (Rauw et al., 1998; Mignon-Grasteau et al., 2005; Oltenacu and Algers, 2005; Jensen et al., 2008; Oltenacu and Broom, 2010).

Temperament and selective breeding for production traits

Comparative and genetic studies in poultry suggest that selective breeding for production traits may also affect temperamental traits such as fearfulness or sociality. For instance, in comparison with Red Junglefowl (the wild ancestor of domesticated layer breeds), modern White Leghorn laying hens behaved less actively in fear and exploration tests, and had a higher motivation to remain close to conspecifics (Schütz et al., 2001; Schütz and Jensen, 2001; Väsänen et al., 2005). Molecular genetic experiments, using progeny from an intercross between Red Junglefowl and White Leghorn lines, demonstrated that, to some extent, quantitative trait loci (QTLs) for such behavioural characteristics were located at the same genomic position as QTLs for production traits like growth and egg weight (Schütz et al., 2002, 2004), thus supporting a genetic basis for the link between selection for increased production and correlated behavioural changes. These findings were interpreted in terms of the resource allocation theory, and were thought to indicate that domesticated laying hens show less energy demanding behaviours than their wild ancestors, allowing them to reallocate the 'saved' energy to production traits (see Jensen, 2001, 2006, 2010; Jensen et al., 2008). Some studies support this idea. Selection for growth in beef cattle, for example, was correlated with slower flight speed (a behavioural measure of cattle temperament, see above), (Burrow and Prayaga, 2004). However, other reports imply an opposite phenomenon, i.e., more active and potentially energy demanding temperamental characteristics were linked with increasing production potential. In pigs, positive correlations were found between lean meat percentage and struggling behaviour during the backtest (Van Erp – van der Kooij et al., 2000, 2003), and between average daily gain and the level of aggression during a resident-intruder test (Cassady, 2007). In a more recent study, average daily gain in pigs

was positively genetically correlated with the number of struggles during the backtest (Velie et al., 2009). Similarly, in some (but not in other) lines of laying hens, continuous selection for higher egg production was associated with increased and more intense behavioural and physiological responses to fearful stimuli, as well as higher levels of feather pecking and cannibalism (Van Hierden et al. 2002; Kjaer and Mench, 2003; Uitdehaag et al., 2008b).

Apparently, reducing the expression of energy demanding behaviours is not the full story when it comes to explaining the effects of selection for higher production on temperamental traits. Van Hierden (2003) offered an interesting alternative hypothesis, and speculated that selection for higher productivity in laying hens may unintentionally target important neurobiological substrates of stress reactivity (e.g., the serotonergic system in the brain), thereby fundamentally changing the (central) neuroendocrine state and, hence, the behaviour and adaptive capacity of the animals. More recently, it was found that feather pecking in progeny from an intercross between Red Junglefowl and White Leghorn strains was linked to an active behavioural pattern in response to challenge (suggestive of a “proactive coping strategy”), as well as early sexual maturation, fast growth, weak bones, and, in males, a high fat accumulation, suggesting that feather peckers have a different resource allocation pattern (Jensen et al., 2005). Possibly, therefore, reallocation of resources and (unpredictable) changes of the neuroendocrine state under the influence of selection for increased production might both be results of this particular selection programme. This, however, remains to be demonstrated. Clearly, the foregoing examples illustrate that the effect of selective breeding on farm animal temperament and fitness is complex, and that, to date, underlying (genetic) mechanisms and pathways remain largely unknown (see Van der Werf, 2007).

Relationships between temperament, fitness and production

Figure 7.2 provides a schematic representation of (partly hypothetical) relationships between the main categories of traits that may affect the fitness of farm animals, including dairy cows. I will use this figure as a framework to further discuss the practical implications of the findings of the present thesis. Arrows (numbered 1 to 3) refer to (largely unknown) pathways and mechanisms underpinning each mutual relationship between categories of traits. Fitness is proposed to be equivalent to “robustness”, and to encompass a range of aspects including health, fertility, longevity and behaviour, e.g., normal, such as feeding, social or maternal behaviours, and abnormal, such as overt

aggressive, harmful or stereotypic behaviours. Defined in this way, fitness determines the extent to which an animal successfully adapts to its environment, and, hence, its state of welfare (see also Moberg, 1987; Wiepkema and Koolhaas, 1993). It is assumed that temperamental traits may affect the fitness of farm animals through the interplay between components of the central nervous, neuroendocrine and immune systems (arrow numbered 1, see Figure 7.2). This was addressed in a previous section of this discussion (see above). Likely through a reallocation of resources (discussed above), selection for production traits may negatively affect the fitness of farm animals in more direct or proximate ways (arrow numbered 2, see Figure 7.2). For example, calcium and other minerals may be diverted from the process of bone formation to that of egg shell formation thereby affecting skeletal strength in laying hens, or bodily reserves may be mobilised to facilitate high milk production to the extent that dairy cows experience weight loss and a negative energy balance, etc. (see Rauw et al., 1998; Sandøe et al., 1999; Oltenacu and Algers, 2005, Veerkamp et al., 2009). It is also hypothesized that selection for production traits may affect temperamental characteristics (arrow numbered 3, see Figure 7.2), including fearfulness, sociality and coping style, because of a tendency to reduce the expression of energy demanding behaviours, and/or through effects on the neuroendocrine state of the animal, which may not necessarily be mediated by a reallocation of resources. This change in temperament, in turn, may again influence fitness (arrow numbered 1, see Figure 7.2). Thus, this “two-stage” route may represent more indirect or ultimate mechanisms by which selection for increased productivity may compromise fitness.

Balanced breeding and temperament

As a means of counteracting negative side effects of breeding for production traits on farm animal fitness and improving their welfare, adaptability and “robustness” it has been widely suggested that more “sustainable” or “balanced” breeding goals, including traits other than those strictly related to production characteristics in the selection index (i.e., multi-trait selection) should be defined (Sandøe et al., 1999; Kanis et al., 2004, 2005; Lawrence et al., 2004; Oltenacu and Algers, 2005; Calus, 2006; Star et al., 2008; Knap, 2009; Ten Napel et al., 2009; Oltenacu and Broom, 2010). Several scenarios have been explored in this respect. Many authors proposed using traits related to fitness (see Figure 7.2, upper right hand side), and, for example, suggested a selection index in dairy cattle based on traits like lameness, mastitis, calving interval and lifespan as measures of health and fertility (see Lawrence et al., 2004; Oltenacu and Algers, 2005). Traits of

this kind have already been recorded in practice, and quantitative evaluations using real data show that, because of antagonistic relationships between production and fitness traits (arrow numbered 3, see Figure 7.2), a trade-off may exist between the costs of lower milk yield and the benefits of a higher health status of cows (Lawrence et al., 2004). Therefore, depending on the rate of genetic change, and the weights applied to each trait, breeding for improved welfare of dairy cows may be profitable overall (Lawrence et al., 2004). Other approaches have also suggested the use of behavioural or temperamental traits for breeding purposes, including, for example, fearfulness, sociality, or aggression (see Faure and Mills, 1998; Jones and Hocking, 1999; Kanis et al., 2004; Boissy et al., 2005a; Star et al., 2008). In terms of Figure 7.2, a breeding index may then consist of traits belonging to each of the three categories. In line with this idea, using temperamental traits in dairy cattle breeding may be one important potential way of implementing the results of the present thesis in practice. Until now however, in contrast to more "classical" fitness traits (see above), the application of temperamental characteristics in farm animal breeding is still largely a matter of theory, open for discussion.

Following the model in Figure 7.2, fitness would determine whether or not an animal is able to successfully adapt to its environment, and this raises the fundamental question of why temperamental traits should be considered in the first place. Ten Napel et al. (2009), for example, suggested focusing on "the results of adaptation" (i.e., fitness), rather than on "the adaptation process itself" when breeding for robustness in cattle. However, one reason to include underlying temperamental traits in a breeding programme would be that these traits are capable of influencing a wide range of biological responses to a broad variety of different challenges (discussed above), and, hence, capture more general and far-reaching features of the adaptive capacity of an animal than individual fitness traits. Moreover, temperamental traits are relatively stable across development, and may therefore predict the risk of a fitness problem before it actually occurs. In a breeding context, this could mean, for example, that selection based on a juvenile temperamental trait might target an adult fitness problem.

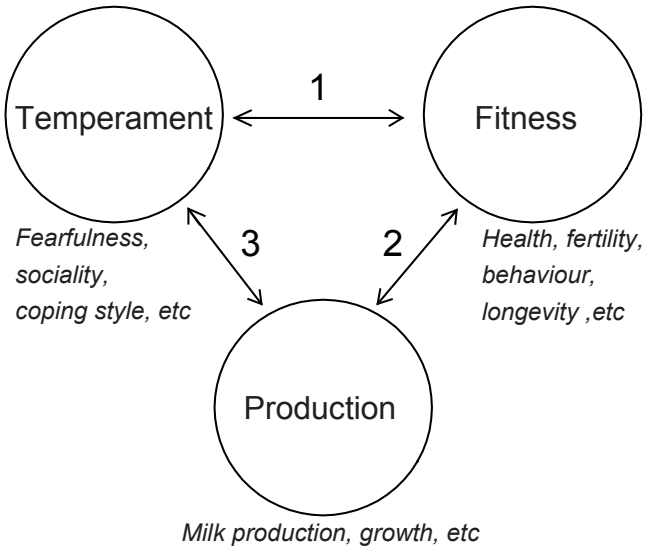

Figure 7.2 Schematic representation of interrelationships between the main categories of traits that determine the "fitness" (or "robustness") of farm animals, including dairy cattle. Fitness encompasses a range of aspects, including health, fertility, behaviour (normal, e.g. feeding, social or maternal behaviours, and abnormal, e.g. overt aggressive, harmful or stereotypic behaviours), and longevity. Fitness defines the extent to which an animal successfully adapts to its environment. Arrows (numbered 1 to 3) refer to (largely unknown) pathways and mechanisms underpinning each mutual relationship. 1. Basic, underlying temperamental (or "adaptive personality") traits, including fearfulness, sociality and coping style, are assumed to affect the fitness of farm animals through the interplay between components of the central nervous, neuroendocrine and immune systems. 2. Persistent selection for production traits, such as milk production or growth, may negatively affect the fitness of farm animals, for example, because of a shift in the allocation of resources. 3. An effect on temperamental traits may provide an additional mechanism by which selection for production traits may ultimately affect the fitness of farm animals. This effect may be the result of a reallocation of resources, and may involve the unintentional co-selection for specific neurobiological characteristics, thereby altering the neuroendocrine state of the animal. In addition to fitness traits, temperamental traits may represent potential selection crititeria to be included in breeding programmes aimed at improving fitness and welfare of farm animals, provided that the (genetic and phenotypic) relationships between production, temperament and fitness traits are elucidated and that the underlying biological mechanisms are better understood.

Appropriate temperament

A second important question that emerges when evaluating the practical feasibility of incorporating temperament traits in farm animal breeding concerns the assessment of the desired or appropriate kind of temperament. Several authors seem to assume *a priori* that selection for temperament should aim at reduced fearfulness or increased sociality (e.g., Faure and Mills, 1998; Jones and Hocking, 1999; Boissy et al., 2005a). Kanis et al. (2004) addressed this issue in a more conceptual way, and described a framework for breeding for improved welfare in pigs, using a thermoregulatory model as a basis. The width of the thermoneutral zone, i.e., the range of ambient temperatures in which little or no behavioural or physiological effort is required to maintain a constant body temperature, was assumed to be positively associated with animal resilience and welfare (Kanis et al., 2004). This principle was extrapolated to other environmental stimuli such as, for example, novel, social, or otherwise (emotionally) demanding situations, and led to the prediction that pigs that exhibit a low average response to a range of challenges (i.e., low aggression, low exploration, low fear, low "nervousness", etc.) experience high resilience and welfare and vice versa (Kanis et al., 2004). From an animal welfare point of view, a desired pig temperament would then by definition refer to animals with low amplitudes of behavioural and physiological responses to challenging situations (i.e., situated on the left-hand side of the distribution presented in Figure 7.1). These assumptions, however, may fall short in a number of ways:

Qualitative dimensions. First, they overlook the fact that behavioural and physiological response patterns of (farm) animals to challenge may also include qualitative dimensions (i.e., the type of response, or coping style, at a given amplitude), that may be important with regard to fitness and welfare (see previous sections of this discussion).

Amplitude of response and adaptation. Second, it is questionable whether low amplitudes of response to stressors are always favourable from an adaptation or fitness point of view. For instance, according to recent theory on robustness of biological systems, the robustness of an organism does not mean that it remains unchanged in response to environmental stimuli; on the contrary, a robust organism is able to mount an adequate and flexible response to a changing environment (Kitano, 2004). This closely agrees with the concept of allostasis, which emphasizes the notions of "maintaining stability through *change*", and providing the animal with the appropriate biological make

up (e.g., behaviourally, physiologically, and neuroendocrinologically) to be able to do so (see McEwen and Wingfield, 2003; Korte et al., 2007; Feder et al., 2009). Recent examples of putative (genetic) relations between temperament and fitness (or "robustness") also support this view, and point to the possibility of a link between a high amplitude and intensity of response to challenge and high fitness. Canario et al. (2009) showed that pigs with a beneficial genetic effect on the growth of pen-mates (using a novel approach to quantify the heritable effects that animals have on their group mates' traits, see Rodenburg et al., 2010, for a review) spent more time fighting and bullying others at mixing, initiated more fights, and also both won and lost more fights. It was suggested that the behaviour of these pigs may benefit pen-mates by speeding the establishment of dominance relationships (Canario et al., 2009). Likewise, Gibbons (2009) compared the behaviour during feeding of daughters (primiparous heifers) of bulls with either a high ("high index") or low ("low index") value of a "robustness index" which was an extension of an existing "Profitable Life Index" in the UK, with increased emphasis on locomotion, somatic cell count, udder health, fertility and lifespan. High index daughters were found to respond more frequently to aggressive interactions (i.e., either by active avoidance or by retaliation) than low index ones (Gibbons, 2009). The present results suggest that a high level of sociality, putatively reflected in a high rate of vocalisations during OF and NOT tests (which equates with a high amplitude of response), may be beneficial to milk ejection at the beginning of lactation in heifers (see chapter 6). Conversely, in other cases low amplitudes of response might be more adaptive than high ones. For example, low aggression and high docility of Limousin heifers during standard encounters with a human handler were genetically associated with good maternal behaviour and high fertility (Phocas et al., 2006).

Genotype x environment interaction. Third, the theory of "adaptive personality differences" or "behavioural syndromes" would predict that the fitness consequences of differences in temperament directly depend on the environmental conditions the animals are exposed to (see Sih et al., 2004a, b; Groothuis and Carere, 2005; Wolf and Weissing, 2010). In other words, from a fitness point of view, a certain response pattern resulting from a particular underlying temperament (involving a specific type or amplitude of response to challenge) could be (highly) beneficial in one environment but less beneficial or even detrimental in another (see also previous sections of this discussion). In population genetic terms, this is described as a "genotype x environment (G x E) interaction" (see Calus, 2006). A number of studies clearly suggest that G x E interactions are also relevant for farm animals, and, hence, for the effects temperamental

traits may have on fitness (arrow numbered 1, see Figure 7.2). Bolhuis et al. (2003, 2006), for example, demonstrated that differences in immune response to antigenic challenge, or in the prevalence of gastric lesions recorded at slaughter, between pigs previously characterised in a backtest as either high or low responders, depended on the housing system (barren versus enriched) the animals were kept in. These findings provide examples of significant temperament x environment interactions at the phenotypic level. In quantitative genetic work in dairy cattle, significant G x E interactions were reported for several putative fitness traits, including body condition score, the number of inseminations before conception, and survival (reflecting whether or not a cow is present on the farm during the next lactation) (Calus et al., 2005).

Collectively, therefore, it is suggested that an appropriate temperament is all about a response to challenge appropriate for the environmental conditions, rather than a certain magnitude or qualitative type of response *per se*. Or, in terms of the model in Figure 7.1, various response patterns situated at different positions in the multidimensional space (or "fitness landscape", see discussion above) may all be associated with an acceptable level of fitness (and welfare), given the right circumstances. At this time, we are still a long way from understanding the biological mechanisms underpinning the relationships between temperament, fitness and selection for enhanced productivity in dairy cattle. Moreover, in contrast to aspects of dairy cow fitness like fertility, health and longevity, temperamental traits, in the sense of the present thesis, have not been recorded in any significant numbers of cows so far. This precludes an immediate application of the current findings in practical dairy cattle breeding.

Practical way forward and prospects

What is needed are studies where the three categories of traits indicated in Figure 7.2 are simultaneously recorded in large numbers of animals in practice, preferably in different environments (e.g., conventional versus organic farms, see Nauta et al., 2006). This will enable quantitative genetic analyses and the estimation of genetic parameters, including phenotypic and genetic correlations between production, temperament and fitness traits. With this information, accurate simulation studies could conceivably identify feasible strategies on the utilization of temperament traits for the improvement of robustness and welfare of dairy cows (see Kanis et al., 2005, and Gourdine et al., 2010, for examples in pigs). However, since the execution of behavioural tests according to the experimental approach described in this thesis is both time- and labour consuming, the practical feasibility of the required large-scale studies may be limited. Thus, there is a

parallel need for the development of simpler and easier methods of recording relevant temperamental traits in dairy cattle. Perhaps the application of sensor-based methods may be helpful in this respect. Schrader (2002), for example, found significant relationships between temperamental characteristics observed during behavioural tests, and measures of dairy cow activity recorded with the use of pedometers (activity monitors) attached to the hind leg (see also Müller and Schrader, 2003). It is tempting to speculate that with the use of sophisticated sensors for monitoring behavioural (e.g., activity) and physiological measures (e.g., temperature, heart rate), aspects of dairy cow temperament might ultimately be tapped on a routine basis (see Berckmans and Guarino, 2008).

Encouragingly, the available data seems to indicate that heritabilities of temperamental characteristics in farm animals, including grazing species such as (dairy) cattle and sheep, are reasonably high, and mostly well within the range reported for production traits. For example, heritabilities of the level of docility of Limousin heifers during standard encounters with a human handler (Le Neindre et al., 1995), the movement of cattle when individually confined on a weighing platform (Schmutz et al., 2001), and vocalization and locomotor responses of sheep to social isolation or fear of human tests (Boissy et al., 2005b; Wolf et al., 2008), all ranged between 0.22 and 0.58. Similar heritabilities for various behavioural (e.g., OF locomotion and vocalization, latency to first contact with a novel object and the time spent in contact with it) and physiological (e.g., heart rate and heart rate variability during confinement in a start box, and the cortisol response to an OF and NO test) measures were obtained in a recent study in dairy calves, using exactly the same protocol for OF and NO testing as described in the present thesis (see chapter 4) (Van Reenen et al., 2008; Eaglen, 2009). Notably, there are also indications that the phenotypic relationship in calves between avoidance of a novel object and the cortisol response to OF and NO tests, (as reported in this thesis (see chapters 3 and 6), also exists at the genetic level (Eaglen, 2009). These findings support the idea that correlated adrenocorticortical and behavioural responses to novelty, and OF locomotion and vocalization reflect genetically mediated temperamental traits in dairy cattle. This merits further study, and may ultimately justify large scale (genetic) research.

CONCLUDING REMARKS

The present thesis reports consistent individual differences in behavioural and physiological responses of dairy cattle to acute experimental stressors, both during the

rearing period (3 weeks to 6-7 months of age), and from the rearing period to (early) adulthood (22 - 29 months of age). In addition, it was demonstrated that dairy heifers exhibited consistent differences in their response to a “real life” challenge such as being machine-milked for the first time, and that part of this variation was explained by differences in earlier behavioural responses to OF and NO tests. These findings significantly strengthen the idea that stress responsiveness in dairy cows is controlled by developmentally stable underlying temperamental characteristics. The multidimensional nature of response patterns to challenges observed in dairy cattle in the current work is supported by reports in other species. Results of the pharmacological validation study, using the fear-reducing agent brotizolam, confirmed the hypothesis that temperament in dairy cows consists of multiple independent dimensions, and identified underlying fearfulness as one of the candidate traits. Other traits likely include sociality and activity (or coping style). Like other (farm) animal species, traits of this kind may affect the fitness of dairy cows in terms of, for example, health, fertility and longevity. In addition to “classical” fitness traits in dairy cows, such as calving interval, lifespan, or measures of lameness and mastitis, temperamental traits may represent additional selection criteria that should be included in breeding programmes aimed at the improvement of “robustness” and welfare. This will require the elucidation of (genetic and phenotypic) relationships between production, temperament and fitness traits, as well as further insight into the biological mechanisms underpinning these relationships. Such knowledge might enable the breeding of dairy cows that are optimally adapted to their environment, including alternative husbandry systems that are intended to improve animal welfare and sustainability (e.g., organic farming systems). Prior to the actual implementation of temperamental traits in breeding programmes, selected behavioural and/or physiological measures of dairy cow temperament might be immediately useful as tools to monitor the potential consequences of selective breeding for adaptive capacity and welfare.

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SAMENVATTING

Achtergrond

Uit onderzoek met een breed scala aan diersoorten, inclusief de mens, blijkt dat er grote verschillen bestaan tussen individuen in de manier waarop met belastende of stressvolle omstandigheden wordt omgegaan. In experimenten waarin dergelijke individuele verschillen worden aangetoond wordt vaak gebruik gemaakt van gecontroleerde belastingen ('challenges'), zoal bijvoorbeeld kortdurende afzondering van soortgenoten of de confrontatie met nieuwe en onverwachte stimuli, en worden zowel gedragsmatige als fysiologische reactiepatronen geregistreerd. Een belangrijke vinding in dit type onderzoek is dat verschillen tussen individuen in gedragsmatige en fysiologische reacties op stressvolle situaties *consistent* zijn. Dat wil zeggen dat karakteristieke reactiepatronen bij herhaling in de tijd hetzelfde blijven, en dat individuen op verschillende belastende of stressvolle situaties op dezelfde manier reageren. Consistentie van zulke individuele verschillen suggereert dat stress-reactiviteit wordt gecontroleerd door stabiele onderliggende eigenschappen ('traits'). In dit verband wordt ook wel gesproken over 'temperament', waarmee de karakteristieke wijze van reageren van het individu op prikkels uit de omgeving wordt bedoeld. Temperament wordt algemeen beschouwd als het erfelijke 'fundament' van de menselijke persoonlijkheid, en heeft betrekking op eigenschappen die mensen delen met (andere) dieren, zoals bijvoorbeeld 'agressiviteit' of 'angstigheid'.

Temperament is niet alleen bepalend voor de manier waarop het individu tijdens de actuele blootstelling aan stressvolle situaties reageert, maar kan ook gevolgen hebben op de langere termijn voor andere belangrijke biologische processen en kenmerken zoals de voortplanting, de gezondheid en de levensduur. Dit betekent dat temperament en 'temperamental traits' van belang kunnen zijn voor de mate waarin landbouwhuisdieren zich succesvol kunnen aanpassen aan de omstandigheden waaronder ze gehouden worden, en daarmee voor hun gezondheid en welzijn.

Doel van het proefschrift

In dit proefschrift is onderzoek beschreven naar temperament van melkkoeien. In het onderzoek is gebruik gemaakt van gedragstests waarbij individuele dieren op een gecontroleerde manier aan kortdurende – en met name psychologische – vormen van belasting worden blootgesteld. Eerder onderzoek heeft laten zien dat volwassen melkkoeien consistent reageren op dergelijke tests, wat de gedachte ondersteunt dat hiermee inderdaad temperament en 'temperamental traits' kunnen worden gemeten. Het

onderzoek beschreven in dit proefschrift beoogde primair om dit idee verder te onderbouwen. De hoofddoelstelling van het huidige proefschrift was om consistentie van individuele verschillen in reacties van melkkoeien te bestuderen op een longitudinale manier, dat wil zeggen van kalf tot koe. Individuele dieren zijn daarom herhaald aan experimentele gedragstests blootgesteld: tijdens de opfokperiode (tot de leeftijd van ongeveer een half jaar), tijdens de periode waarin de dieren voor het eerst drachtig waren, en tijdens de eerste lactatie. Het onderzoek beschreven in dit proefschrift had verder tot doel om het begrip en de interpretatie van reactiepatronen van melkkoeien op gedragstests te verbeteren door het gelijktijdig met gedragsparameters registreren van fysiologische en neuroendocriene parameters, en door de toepassing van een farmacologische methode voor de validatie van angsttests. Tenslotte is onderzoek gedaan naar de betekenis van temperament voor de manier waarop melkkoeien omgaan met een stressvolle situatie zoals die in de normale houderij voorkomt.

De resultaten

In het experiment beschreven in **hoofdstuk 2** werden 25 vaarskalveren blootgesteld aan dezelfde set van vier gedragstests op drie leeftijden, namelijk 3, 16 en 29 weken. De gedragstests waren een ‘open field test’, waarbij een kalf gedurende 15 minuten werd afgezonderd van haar soortgenoten in een testruimte, een ‘novel object test’, waarbij een kalf in een testruimte werd geconfronteerd met een nieuw voorwerp, een ‘restraint test’, waarbij een kalf gedurende korte tijd in afzondering werd aangebonden, en een ‘human approach test’, waarbij een kalf in een testruimte werd geconfronteerd met een persoon. In elke test werd de gedagsreactie van de kalveren nauwkeurig gekwantificeerd. Met behulp van statistische methoden werd nagegaan (i) in hoeverre gedragsreacties binnen leeftijden tussen verschillende tests met elkaar correleerden (consistentie tussen tests), en (ii) in hoeverre gedragsreacties tussen leeftijden met elkaar correleerden (consistentie in de tijd). Het bleek dat binnen leeftijden de gedragingen van de kalveren konden worden samengevat in vier aparte clusters van onderling gecorreleerde gedragingen: vocalisaties (loeien), locomotie (lopen en bewegen tijdens aanbinden), interactie met het nieuwe object (latentietijd tot eerste contact, hoeveelheid contact met object), en interactie met de persoon (latentietijd tot eerste contact, hoeveelheid contact met persoon). Verschillen tussen kalveren in locomotie, vocalisaties, en interactie met het nieuwe object waren consistent tussen 16 en 29 weken. Dit suggereerde dat deze gedragsreacties worden gecontroleerd door stabiele

onderliggende eigenschappen, waarschijnlijk behorend tot het temperament van (opgroeiende) melkkoeien.

In **hoofdstuk 3** werden twee van de eerder gebruikte tests, de open field test en de novel object test, verder onderzocht, en werden naast het gedrag ook fysiologische (hartslag) en neuroendocriene (stresshormoon cortisol, reactivititeit van de hypothalamus-hypofyse-bijnier as) parameters waargenomen. Twintig vaarskalveren werden individueel onderworpen aan de twee gedragstests op een leeftijd van 3, 13 en 26 weken. Opnieuw werden correlatiepatronen tussen de gemeten variabelen geanalyseerd, binnen en tussen leeftijden. Kalveren die snel contact maakten met het nieuwe object (korte latentietijd tot eerste keer aanraken) en langdurig met het object interacteerden hadden een lagere cortisol respons, zowel tijdens de novel object test als tijdens de open field test, en een hogere hartslag reactie tijdens de fase vlak voor het begin van de open field test wanneer de dieren zich gedurende 3 minuten in een zogenaamde startbox bevonden. Individuele verschillen in dit reactiepatroon waren consistent tussen alle drie de leeftijden. Lopen en loeien waren, net als in hoofdstuk 2, onderling niet gecorreleerd, en hielden ook geen verband met de cortisol respons of de (snelheid van) interactie met het nieuwe object. Verschillen tussen kalveren in loeien waren consistent tussen 13 en 26 weken.

De resultaten van hoofdstuk 2 en hoofdstuk 3 leidden tot de hypothese dat aan de reactiviteit van kalveren op de gebruikte gedragstests tenminste drie onafhankelijke onderliggende temperament-gerelateerde eigenschappen ten grondslag liggen: (i) activiteit of ‘coping style’ (gerelateerd aan lopen/bewegen), (ii) de neiging om dicht bij soortgenoten in de buurt te zijn of ‘sociality’ (gerelateerd aan loeien), en (iii) de mate van angstigheid of ‘fearfulness’ (gerelateerd aan de cortisol respons en de interactie met het nieuwe object). Deze hypothese is getoetst in **hoofdstuk 4**, door aan kalveren voorafgaand aan een gecombineerde ‘open field – novel object test’ een angstremmer (brotizolam) toe te dienen. In vergelijking met dieren die een placebo kregen toegediend lieten kalveren die met de angstremmer waren behandeld een kortere latentietijd tot eerste contact met het nieuwe object, een langere tijd in contact met het object en een snellere daling van het gehalte aan cortisol in het bloed na afloop van de test zien. Toediening van brotizolam had geen effect op lopen en op loeien. Deze bevindingen bevestigden het idee dat de gedragsreactie van kalveren ten aanzien van een nieuw object, en de daarmee gecorreleerde cortisol respons, samenhangen met ‘angstigheid’ en dat locomotie en vocalisatie andere temperament-gerelateerde eigenschappen reflecteren, zoals bijvoorbeeld ‘coping style’ en ‘sociality’.

Het voor het eerst gemolken worden in een melkstal is voor melkkoeien een nieuwe en mogelijk stressvolle ervaring. Vanuit de gedachte dat temperament een rol kan spelen in de reactie van melkkoeien op deze situatie is in **hoofdstuk 5** gekeken naar individuele verschillen tussen dieren gedragsmatige, fysiologische en neuroendocriene reacties op het gemolken worden. Bij 23 vaarzen werden metingen gedaan tijdens de allereerste melking na afkalven, tijdens de vijfde melking en tijdens een melking ongeveer 130 dagen na afkalven. Tijdens de eerste en de vijfde melking na afkalven bleken dieren die hun melk moeilijk lieten schieten ook de individuen te zijn met een lage afgifte van het hormoon oxytocine tijdens het melkproces, een lage melksnelheid, en een hoge hartslag reactie tijdens het voorbehandelen van het uier door de melker. Individuele verschillen in dit reactiepatroon waren consistent tussen de eerste en de vijfde melking. Rond dag 130 verliep de melkafgifte bij alle dieren ongestoord. De gedragsreacties van koeien op het gemolken worden (het aantal stappen, en het aantal trappen met de achterpoten) waren ook sterk individu-gebonden, maar correlereerden niet met de melkafgifte. Op grond van deze resultaten werd verondersteld dat de mate van angstigheid (voor de melker en voor andere nieuwe prikkels tijdens het melkproces) de melkafgifte van vaarzen aan het begin van de lactatie negatief beïnvloedt.

Het onderzoek beschreven in **hoofdstuk 6** was bedoeld om meer aanwijzingen te verkrijgen voor een mogelijke relatie tussen temperament en de reactie van melkkoeien op een 'real life challenge' zoals het voor het eerst gemolken worden. De 23 vaarzen waarbij de reactie op het gemolken worden was bestudeerd (hoofdstuk 5) waren dezelfde dieren die als kalf een viertal gedragstests hadden ondergaan (hoofdstuk 2). Een analyse van correlaties tussen juveniele en adulte reactiepatronen op potentieel stressvolle situaties wees uit dat dieren die als kalf vaker loeiden tijdens gedragstests als vaars een betere melkafgifte lieten zien tijdens de eerste machinale melking na afkalven. Dit zou erop kunnen wijzen dat de motivatie om dicht bij soortgenoten te zijn ('sociality'), waarvan het veelvuldig loeien tijdens gecontroleerde gedragstests een uiting zou kunnen zijn, van invloed is op de melkafgifte aan het begin van de lactatie van vaarzen. Dieren die graag dicht in de buurt van soortgenoten verblijven zouden dan de individuen zijn die in een stressvolle situatie het meest gebaat zijn bij sociaal contact, en daardoor de minste negatieve gevolgen van die situatie (zoals een door stress geïnduceerde belemmering van de melkafgifte) ervaren.

De dieren die als kalf waren onderworpen aan gedragstests (hoofdstuk 2 en hoofdstuk 3) zijn ook tijdens de periode dat ze voor het eerst drachtig waren, of tijdens de eerste lactatie, aan één of meer van dezelfde gecontroleerde tests blootgesteld.

Vervolgens is geanalyseerd in hoeverre reacties tijdens de opfok die tijdens latere fasen van het leven voorspellen, en de resultaten van deze analyses worden eveneens in hoofdstuk 6 gerapporteerd. In het reactiepatroon van melkkoeien op de gebruikte gedragstests waren ook op oudere leeftijd drie onafhankelijke clusters variabelen waarneembaar, gerelateerd aan, respectievelijk, lopen/bewegen, loeien en de cortisol respons. Individuele verschillen in lopen/bewegen, in loeien, of in de cortisol respons (en de daarmee gecorreleerde gedragsreactie ten aanzien van het nieuwe object) gemeten bij kalveren op een leeftijd van ongeveer een half jaar bleken over het algemeen significant te correleren met diezelfde verschillen waargenomen op oudere leeftijd.

Discussie en conclusie

De resultaten van dit proefschrift zijn een belangrijke ondersteuning voor het idee dat met behulp van gedragstests zoals gebruikt in dit proefschrift stabiele temperament-gerelateerde eigenschappen bij melkkoeien kunnen worden gemeten.

In **hoofdstuk 7** worden de bevindingen bediscussieerd tegen de achtergrond van de belangrijkste theorieën in de wetenschappelijke literatuur met betrekking tot individuele verschillen en temperament. Tevens wordt ingegaan op de mogelijke betekenis van de uitkomsten van dit proefschrift voor de praktijk. De suggestie dat het temperament van melkkoeien uit meerdere onderliggende eigenschappen bestaat, waaronder activiteit of 'coping style', 'fearfulness', en 'sociality', wordt ondersteund door een groot aantal bevindingen bij andere diersoorten. Beargumenteerd wordt dat een aan het temperament gerelateerd profiel ('temperamental profile'), dat door meerdere van dergelijke eigenschappen wordt bepaald, het individu meer of minder gevoelig kan maken voor de (negatieve) gevolgen van belastende omstandigheden in de omgeving, bijvoorbeeld voor wat betreft de vruchtbaarheid, de gezondheid of de levensduur. Aspecten als vruchtbaarheid, gezondheid en levensduur van (landbouwhuis)dieren worden in de wetenschappelijke literatuur ook wel onder een gemeenschappelijke noemer gedefinieerd als 'robuustheid' of 'fitness'. Ook wordt in hoofdstuk 7 aannemelijk gemaakt dat, net als bij andere diersoorten, temperament-gerelateerde eigenschappen bij melkkoeien voor een deel erfelijk bepaald zijn. Een mogelijke toepassing van de bevindingen van dit proefschrift zou er daarom uit kunnen bestaan dat 'temperamental traits' worden gebruikt als additionele selectiecriteria in de fokkerij met het doel om meer 'robuuste' koeien te verkrijgen, met uiteindelijk een beter welzijn. Voor het zover is, is meer kennis nodig over onderlinge (genetische) relaties tussen (i) productiekenmerken (zoals melkproductie), (ii) temperament-gerelateerde kenmerken, en (iii) een breed scala

aan ‘fitness’ kenmerken (gerelateerd aan, bijvoorbeeld, vruchtbaarheid, gezondheid, en levensduur). Bovendien zal naar methoden gezocht moeten worden waarmee temperament van melkkoeien op een veel eenvoudiger manier gemeten kan worden dan met behulp van de in dit proefschrift gebruikte gedragstests.

DANKWOORD

In de experimenten die tot dit proefschrift hebben geleid heb ik zogenaamd “longitudinaal” onderzoek gedaan bij melkkoeien. Dat wil zeggen dat ik dieren heb gevolgd van kalf tot koe. Ik heb mij o.a. de vraag gesteld of het temperament van jonge dieren voorspellend is voor het temperament op oudere leeftijd. De totstandkoming van dit proefschrift is uiteindelijk ook een behoorlijk longitudinale onderneming geworden. De eerste plannen om te promoveren dateren van 1992, toen ik net was aangesteld als junior onderzoeker bij het Instituut voor Veeteeltkundig Onderzoek in Zeist. In die periode ben ik vol enthousiasme begonnen met de eerste experimenten met opgroeiende kalveren. Destijds was ik er van overtuigd dat ik over het juiste temperament beschikte om een dergelijke uitdagende onderneming binnen een redelijke termijn tot een goed einde te brengen. Inmiddels is het 2012 en het was maar de vraag of mijn eerder veronderstelde gunstige temperament voldoende consistent was om mij ook op latere, ik mag wel zeggen middelbare, leeftijd in staat te stellen om een wetenschappelijk proefschrift met succes af te ronden.

Na 20 jaar is het proefschrift dan eindelijk klaar en dat zou kunnen betekenen dat ik zelf het levende bewijs ben van het bestaan van lange-termijn consistentie in “wetenschappelijk” temperament. Het zou echter ook zo kunnen zijn dat er met het ouder worden juist veel veranderingen zijn opgetreden, en dat de “wijsheid pas met de jaren is gekomen”.

Hoe dan ook, naast mijn eigen, al dan niet consistente, temperament is het toch vooral ook de inzet en hulp van anderen geweest die er voor heeft gezorgd dat ik nu een formele punt kan zetten achter het “project proefschrift schuine streep promotie” (voor de goede verstaander: op het “p-woord” rust voortaan in mijn aanwezigheid geen taboe meer). Dit is de plaats om alle mensen die een rol hebben gespeeld bij mijn promotie, en bij het vele onderzoekswerk dat daarvoor is gedaan, zeer hartelijk te bedanken!

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handling, coördinerend vermogen, vooruitziende blik, gevoel voor kalveren en koeien ("Friese boerenzoon"…), etc., hadden we de vaak complexe experimenten nooit tot een goed einde kunnen brengen. Gert, wij kennen elkaar al vanaf 1980, toen we allebei studeerden in Wageningen. Jij weet als geen ander waar mijn passie voor onderzoek vandaan komt en wat er allemaal op mijn pad is gekomen gedurende de afgelopen 32 jaar. Wij hebben vele hoogtepunten (en een enkel dieptepunt) samen beleefd en jouw rol als paranimf bij mijn promotie beschouw ik als een nieuw hoogtepunt in onze vriendschap.

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Kees
Februari 2012

CURRICULUM VITAE

Cornelis Gerrit (Kees) van Reenen werd geboren op 10 maart 1961 in Onderdendam (gemeente Bedum), in de provincie Groningen, en groeide vanaf 1964 op in Voorthuizen (tot 1970) en Harderwijk. In 1979 werd het VWO-diploma behaald aan het Christelijk College Nassau Veluwe in Harderwijk. Van jongs af aan had hij een grote wens om dierenarts te worden, maar in 1979 werd hij uitgeloot voor de studie Diergeneeskunde. Als aanvankelijk tijdelijke oplossing werd in 1979 begonnen met de studie Zoötechniek (veeteeltkunde) aan de toenmalige Landbouwuniversiteit in Wageningen. Na nog drie vergeefse pogingen om in te loten voor Diergeneeskunde besloot hij om definitief in Wageningen te blijven. In 1986 werd het kandidaatsexamen behaald, en in 1988 studeerde hij *cum laude* af op een verzwaard hoofdvak Ethologie, een hoofdvak Gezondheids- en Ziekteleer der Huisdieren, en stages bij het Laboratorium voor Pathologie van het RIVM in Bilthoven, en Statens Husdyrbrugsforsøg in Foulum (Denemarken). Na zijn militaire dienst werkte hij als beleidsmedewerker Veterinaire Dienst bij het toenmalige Ministerie van Landbouw, Natuurbeheer en Visserij. In 1992 is hij begonnen als junior-onderzoeker bij het toenmalige Instituut voor Veeteeltkundig Onderzoek (IVO) in Zeist. Na een fusie met andere onderzoeksinstituten is het IVO verplaatst naar Lelystad en uiteindelijk overgaan in de Animal Sciences Group (ASG) van Wageningen Universiteit en Research Center (WUR). De experimenten die de basis hebben gevormd voor dit proefschrift zijn verspreid over een langere periode uitgevoerd, eerst gedurende korte tijd in Zeist, en daarna op een melkveeproefbedrijf in Lelystad. Bij de ASG is hij momenteel werkzaam als senior onderzoeker / projectleider bij het onderdeel Livestock Research, Afdeling Dierenwelzijn, in Lelystad.

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